



Regulation of late and very late gene expression in both lytic and persistent baculovirus infections

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Abstract

Autographa californica nucleopolyhedrovirus (AcMNPV) is an insect-specific pathogen, with a biphasic replication cycle generating both budded viruses (BVs) and occlusion bodies (OBs). While BVs are released from the cell membrane mainly in the late phase of infection, OBs are produced in the subsequent very late phase and released after cell lysis. A characteristic of this very late phase of infection is the hyperexpression of the viral genes *p10* and *polyhedrin* (*polh*), which are nonessential *in vitro*. While polyhedrin is a highly robust protein accumulating in the nucleus of virus-infected cells surrounding the virions and offering a degree of protection, the precise role of P10 is still unclear. It appears to have various roles at the end of the viral replication cycle such as nuclear stability and nuclear disintegration. Importantly, this stage has been utilised to produce recombinant proteins (e.g. vaccines). However, persistent baculovirus infections are also known. They are maintained throughout the life cycle of the host without causing mortality and insect liquefaction for polyhedra dissemination. The molecular mechanism of how the fine balance of host and virus gene expression is maintained remains elusive. Therefore, the aim of this thesis was to determine how late and very late genes in both lytic and persistent baculovirus infections are regulated.

To investigate the regulation of AcMNPV gene expression the effect of the candidate genes *cathepsin*, *chitinase A*, *late expression factor 2* (*lef-2*) and *vp39* were analysed on very late gene expression in overt infections by various approaches such as gene knock-outs, mutagenesis studies or RNA interference.

The late proteins cathepsin and chitinase A are viral enzymes required for liquefaction of the viral host *in vivo*. Moreover, cathepsin was implicated to be involved in cell lysis. It could be determined that P10 was specifically cleaved by the cathepsin independently of chitinase A. Moreover, it appears that processed P10 was crucial for nuclear disintegration and polyhedra release.

Lef-2 is an early viral gene required for AcMNPV DNA replication. Additionally, it was previously hypothesised that it may be important for very late gene expression. Mutagenesis studies on LEF-2 confirmed that different protein domains are crucial during DNA replication and BV production, while its role in very late gene expression remains elusive.

VP39 is a structural protein and the major capsid protein in AcMNPV. However, it was also speculated that it may be involved in *p10* and *polh* expression. Knock-down of *vp39* and subsequent analysis of the effect on mRNA abundances confirmed not only the down regulation of very late gene expression but showed moreover a global effect on viral gene transcription.

Additionally, a more global approach by RNA-sequencing was performed to compare host and virus gene expression in both lytic and persistent AcMNPV infections utilising an *in vitro* system, the *Trichoplusia ni* C20 cell line that supports the continuous replication of low-level infection of AcMNPV lacking *p10*. It could be determined that all AcMNPV genes were expressed in C20 cells at low levels but without distinct temporal phases or shut down of host gene expression. Additionally, transcription of genes required for specific cellular pathways such as endocytosis were altered compared to AcMNPV-infected *T. ni* Hi5 cells.

Recent investigations to recreate an insect cell line harbouring a persistent baculovirus were unsuccessful. It was proposed that improved BV production was too high to allow a persistent state of virus replication to be achieved. Hence, a virus with an impaired BV production, AcLEF-2^{C84A}, was utilised to generate a new *T. ni* Hi5 cell line containing a persistent baculovirus. Preliminary characterisation of this cell line (AcLEF-2^{C84A}/Hi5) revealed similarities to the C20 cell line with respect to its resilience and continuous production of BV.

Oral and poster presentations

Much of the work described in this thesis has been presented at national and international meetings and conferences.

Talks

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Bannach, C., King, L. A. and Possee, R.D. (2016), The transition between late and very late gene expression in baculovirus infected cells, with application in the development of expression vectors for VLP-based vaccines. School of Life Sciences Annual Graduate College Event, Oxford Brookes University, Oxford, UK.

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For my mum

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Abbreviations

α	Anti
A	Adenosine
AcMNPV	Autographa californica nucleopolyhedrovirus
BEVS	Baculovirus expression vector system
β-Gal	Beta-galactosidase
bp	Base pair
BrdU	5'-bromodeoxyuridine
BTI	Boyce Thompson Institute
BV	Budded virus
C	Cytosine
<i>cath</i>	<i>Cathepsin</i>
cDNA	Complementary DNA
<i>chit</i>	<i>Chitinase</i>
<i>Cpe</i>	Cytophasic effect
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated
C_T	Threshold cycle
C-terminus	Carboxyl-terminus
d	Day
Da	Dalton
DGE	Differential gene expression
DIF	Defective interfering particle
ds	Double-stranded
DNApol	DNA polymerase
dNTP	Deoxynucleotide triphosphate mix
eGFP	Enhanced Green Fluorescent Protein
EM	Electron microscope
EMA	European Medicines Agency
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
EU	European Union
FC	Fold change
FCS	Fetal calf serum
FDA	Food and Drug Administration
FP	Few polyhedra
h	Hour
HA	Hemagglutinin
HDAC	Histone deacetylase
HPV	Human Papillomavirus
HSV	Herpes Simplex Virus
HsNV	Heliothis zea nudiviruses
G	Guanine
G phase	Gap phase
GlcNAc	β -1,4-N-acetylglucosamine
GST	Glutathione-S-transferases
GV	Granulovirus
HPV	Human Papillomavirus
HSV	Herpes Simplex Virus
IFN-β	Beta Interferon
IVRG	Insect virus research group
JAK/STAT	Janus kinase/signal transducers and activator of transcription
k	Kilo
LEF	Late expression factor
m	milli
M	Molar
M phase	Mitosis phase
MAPK	Mitogen-activated protein kinase
min	Minute
mRNA	Messenger RNA
MNPV	Multiple nucleopolyhedrovirus
MOI	Multiplicity of infection
MVB	Multivesicular body
n	nano
NCBI	National Center for Biotechnology Information
NFκB-IκB	nuclear factor kappa B- inhibitor of kappa B
NPC	Nuclear pore complex
NPV	Nucleopolyhedrovirus

nt	Nucleotide
N-terminus	Amino-terminus
OB	Occlusion body
ODV	Occlusion derived virus
orf	Open-reading frame
p	passage
PAS	Polyadenylation signal
PCR	Polymerase chain reaction
p.i.	Post infection
PIF	<i>Per os</i> infectivity factor
Polh	Polyhedrin
p.s.	Post settling
qPCR	Quatitative PCR
RNAi	RNA-interference
RPKM	Reads per kilobase per transcript per million mapped reads
rRNA	Ribosomal RNA
RT-PCR	Real time PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S phase	Synthesis phase
Sf	<i>Spodoptera frugiperda</i>
siRNA	Small interfering RNA
SLE	St Louis encephalitis
SNARE	soluble N-ethylmaleimide-sensitive factor receptors
ss	Single-stranded
T	Thymine
<i>T. ni</i>	<i>Trichopulsia ni</i>
TREX	Transcriptional export complex
tRNA	Transfer RNA
ts	Temperature sensitive
U	Units
US	United Stated
UTR	Untranslated region
TNCL	<i>T. ni</i> Hi5 cell line virus
TSS	Transcriptional start site
v	Volume
VLP	Virus-like particle
VLD1	Very late deficiency virus 1
VS	Virogenic stroma
w	Weight
wt	Wild-type
X-Gal	5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Amino acid code

Amino Acid	Three letter coder	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Meth	M
Phenylalanine	Phe	Y
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Valine	Val	V

Chapter One:

Introduction

1.1 Introduction

Viruses are small nucleic acid containing infectious agents. They appear in a wide variety of sizes usually between 20-400 nanometres (nm) and a variety of shapes from simple to complex and from helical to icosahedral. A protein coat, the so-called capsid, encloses the nucleic acids of most viruses protecting them from environmental stress factors. Viruses depend absolutely on the cellular machinery of their host to replicate. All cellular forms from prokaryotes to eukaryotes are known to be susceptible to viruses (reviewed by Modrow *et al.*, 2010).

Virus infection in mammalian species can cause a broad range of diseases from the more trivial such as the common cold (e.g. rhinovirus) to lethal infections (e.g. rabies virus). Moreover, some viruses are associated with the cause of cancer (e.g. Human Papillomavirus (HPV) 16 and 18). Although vaccines can protect against particular virus diseases (e.g. measles) and resulted in the extinction of smallpox, epidemic and pandemic outbreaks of other viruses still arise regularly. The worst recent outbreaks include the Zika virus epidemic in South America and the Caribbean (2015-2016), the Ebola virus epidemic in Western Africa (2014) and the pandemic flu ("swine flu", 2009; WHO Disease Report).

Therefore, it is important to gain a more detailed knowledge of viruses and virus infection but not only because they are the cause of many diseases: Viruses are simple systems that are easy to manipulate. Hence, they have been used as vectors to express recombinant proteins (e.g. adenoviruses, lentiviruses or baculoviruses). However, virus expression vectors such as the baculovirus system still have their limitations: Baculovirus infection is a lytic process ultimately leading to cell death (Introduction 1.6.1). Therefore, recombinant proteins are not produced continuously and require the frequent infection of fresh cultures. Additionally, expression levels between individual proteins alter constantly, whereby some proteins are produced to higher yields. For example, cytoplasmic proteins are usually expressed higher than glycoproteins destined for the plasma membrane. A crucial factor may also be the time point of recombinant protein expression during the viral replication cycle (reviewed by van Oers, 2011; van Oers *et al.*, 2014). The reasons for the variations in recombinant protein yields are not fully understood yet and may occur either on transcriptional, translational or posttranslational levels. Nevertheless, a major advantage of baculoviruses is that they exclusively infect arthropods but do not spread to humans. Moreover, they are able to transduce mammalian cells for the expression of genes under the control of mammalian promoters (Hofmann *et al.*, 1995).

Viral vectors have been successfully applied for clinical use. For example, lentivirus vectors were approved for cellular therapy for young patients with acute lymphoblastic

leukaemia in the United States (US) by the US Food and Drug Administration (FDA; reviewed by Ledford, 2017). However, owing to safety concerns about adeno- and lentiviruses causing serious illnesses in humans and the oncogenic potential of lentivirus vectors, they often do not gain medical approval (Shimizu *et al.*, 2004; reviewed by Schlimgen *et al.*, 2016). Thus, *Autographa californica* nucleopolyhedrovirus (AcMNPV) as a member of the family of the insect-specific *Baculoviridae* has been increasingly used as a viral vector e.g. for gene therapy to treat or prevent diseases (e.g. baculovirus vectors for human prostate cancer gene therapy; Swift *et al.*, 2013) or for vaccine generations (e.g. baculovirus vectors for vaccines against HPV16, 18 or Influenza, Introduction 1.7; Harper, 2008; Braunagel *et al.*, 2009; Cox and Hashimoto, 2011; Treanor *et al.*, 2011). Hence, it is crucial to study and understand baculovirus gene expression in more detail to further improve their applications as safe expression vector.

1.2 The Baculoviridae

The members of the *Baculoviridae* comprise a diverse family of arthropod-specific pathogens of the orders Diptera, Hymenoptera and Lepidoptera with over 600 host species (Martignoni and Iwai, 1986). Baculoviruses were recognised as important contributors in the regulation of natural insect populations as their infection can cause the mortality of their host. Therefore, they have been utilised for the suppression of pests mainly to protect crops but also forest and pastures (reviewed by Szewczyk *et al.*, 2006).

The name baculovirus originates from the Latin word *baculum*, which is translated as “stick” or “rod” and thus describes the rod-shaped form of the virions (Vago *et al.*, 1974; Fraser, 1986). The members of this family are characterised by large enveloped virions containing a supercoiled, double-stranded (ds) DNA. The baculovirus nucleocapsids vary between 230-385 nm in length and 40-60 nm in diameter. Their genomes consist of non-overlapping open-reading frames (orfs) and range in size from 90-180 kilobase pair (kbp) with a set of 38 conserved core-genes, which most likely play an important role during their replication cycle (reviewed by Blissard and Rohrmann, 1990; Rohrmann, 1992, 2013).

1.3 Classification and Nomenclature

A prominent feature of baculoviruses is the presence of OBs (Fig. 1.1A). Based on their OB morphology, they are divided into granuloviruses (GV) or nucleopolyhedrovirus (NPV) (Murphy and Pringle, 1995). Members of the GVs produce small granular oval-shaped OBs called granules (0.2-0.4 µm) usually holding a single virion. In contrast, NPsVs generate larger OBs also referred to as polyhedra (0.2-2 µm). Polyhedra contain either single (SNPV) or multiple (MNPV) nucleocapsids. Virions inside the OBs are embedded in

a paracrystalline matrix comprising either granulin (GV) or polyhedrin (reviewed by Blissard and Rohrmann, 1990; Rohrmann, 1992, 2013).

With the increase in molecular tools and knowledge of the nucleotide sequences of their genomes, the morphology-based classification of baculoviruses needed to be adapted. Thus, it was proposed to characterise baculoviruses based on phylogenetic approaches into alpha- (lepidopteran-specific NPVs divided in group I and group II NPVs), beta- (lepidopteran-specific GVs), gamma- (hymenopteran-specific NPVs) and deltaviruses (dipteran-specific NPVs; Table 1.1; Jehle *et al.*, 2006). The alphabaculovirus group is by far the largest and contains the baculovirus prototype and most well studied AcMNPV (Miller and Dawes, 1979; Smith and Summers, 1979; Lübbert *et al.*, 1981; Possee, 1986; Possee *et al.*, 1991).

Baculoviruses are usually named after the initial host they were isolated from. Historically, the names contained the initials of the host genus, the host species and the virus OB morphology. As a consequence, many baculoviruses are very closely related although their names are unique, e.g., the virus isolates from *Autographa californica* (AcMNPV), *Anagrapha falicifera* (AfMNPV), *Trichoplusia ni* (TnMNPV), *Galleria mellonella* (GmMPV), *Rachiplusia ou* (RoNPV) and *Plutella Xylostella* (PlxyNPV) (Smith and Summers, 1979; Summers *et al.*, 1980; Brown *et al.*, 1985; Chen *et al.*, 1996; Yanase *et al.*, 2000; Harrison *et al.*, 2012).

As more baculovirus species became isolated, the abbreviations of different species overlapped. As a consequence, the nomenclature was changed to the first two-letters of the host genus and the host species, e.g. *Agrotis ipsilon* MNPV as AgipMNP. Confusingly, the original descriptors for some viruses (e.g. AcMNPV, *Orgyia pseudotsugata* MNPV as OpMNPV) have been kept in parallel with the new (Table 1.1).

Table 1.1 Completely sequenced baculovirus genomes*

Name	Short Name	Accession No
Alphabaculovirus		
<i>Adoxophyes honmai</i> nucleopolyhedrovirus	AdhoNPV	NC_004690
<i>Adoxophyes orana</i> nucleopolyhedrovirus	AdorNPV	NC_011423
<i>Agrotis ipsilon</i> multiple nucleopolyhedrovirus	AgipMNP	NC_011345
<i>Agrotis segetum</i> nucleopolyhedrovirus A	AgseNPV_A	NC_007921
<i>Agrotis segetum</i> nucleopolyhedrovirus B	AgseNPV_B	NC_025960
<i>Antheraea pernyi</i> nucleopolyhedrovirus	ApNPV	NC_008035
<i>Anticarsia gemmatilis</i> multiple nucleopolyhedrovirus	AgNPV-37	NC_031761
<i>Anticarsia gemmatilis</i> nucleopolyhedrovirus	AgNPV-2D	NC_008520
<i>Apocheima cinerarium</i> nucleopolyhedrovirus	ApciNPV	NC_018504
<i>Autographa californica</i> nucleopolyhedrovirus	AcMNPV	NC_001623
<i>Bombyx mori</i> nucleopolyhedrovirus	BmNPV	NC_001962
<i>Buzura suppressaria</i> nucleopolyhedrovirus	BusuNPV	NC_023442
<i>Catopsilia pomona</i> nucleopolyhedrovirus	CapoNPV	NC_030240
<i>Choristoneura fumiferana</i> DEF multiple nucleopolyhedrovirus	CfMNPV_Def	NC_005137
<i>Choristoneura fumiferana</i> multiple nucleopolyhedrovirus	CfMNPV	NC_004778
<i>Choristoneura murinana</i> nucleopolyhedrovirus	ChmuNPV	NC_023177

Table 1.1 Continued: Completely sequenced baculovirus genomes*

<i>Choristoneura rosaceana</i> alphabaculovirus	CrNPV	NC_021924
<i>Chrysodeixis chalcites</i> nucleopolyhedrovirus	CcNPV	NC_007151
<i>Clanis bilineata</i> nucleopolyhedrovirus	CbNPV	NC_008293
<i>Condylorrhiza vestigialis</i> MNPV	CoVeNPV	NC_026430
<i>Ectopis obliqua</i> nucleopolyhedrovirus	EoNPV	NC_008586
<i>Epiphyas postvittana</i> nucleopolyhedrovirus	EpNPV	NC_003083
<i>Euproctis pseudoconspersa</i> nucleopolyhedrovirus	EupsNPV	NC_012639
<i>Helicoverpa armigera</i> nucleopolyhedrovirus	HearNPV	NC_003094
<i>Hemileuca</i> sp. nucleopolyhedrovirus	HespNPV	NC_021923
<i>Hyphantria cunea</i> nucleopolyhedrovirus	HyCuNPV	NC_007767
<i>Lambdina fiscellaria</i> nucleopolyhedrovirus	LafiNPV	NC_026922
<i>Leucania separata</i> nucleopolyhedrovirus	LsNPV	NC_008348
<i>Lymantria dispar</i> multiple nucleopolyhedrovirus	LdMNPV	NC_001973
<i>Lymantria xyliana</i> MNPV	LyxyMNPV	NC_013953
<i>Mamestra brassicae</i> multiple nucleopolyhedrovirus	MabrMNPV	NC_023681
<i>Mamestra configurata</i> nucleopolyhedrovirus A	McNPV_A	NC_003529
<i>Mamestra configurata</i> nucleopolyhedrovirus B	McNPV_B	NC_004117
<i>Maruca vitrata</i> nucleopolyhedrovirus	MvNPV	NC_008725
<i>Orgyia leucostigma</i> nucleopolyhedrovirus	OINPV	NC_010276
<i>Orgyia pseudotsugata</i> multiple nucleopolyhedrovirus	OpMNPV	NC_001875
<i>Peridroma alphabaculovirus</i>	PespNPV	NC_024625
<i>Pseudoplusia includens</i> SNPV IE	PsinSNPV	NC_026268
<i>Spodoptera exigua</i> multiple nucleopolyhedrovirus	SpexMNPV	NC_002169
<i>Spodoptera frugiperda</i> multiple nucleopolyhedrovirus	SfMNPV	NC_009011
<i>Spodoptera litura</i> nucleopolyhedrovirus	SpliNPV	NC_003102
<i>Spodoptera litura</i> nucleopolyhedrovirus II	SpliNPV_II	NC_011616
<i>Sucra jujuba</i> nucleopolyhedrovirus	SujuNPV	NC_028636
<i>Thysanoplusia orichalcea</i> nucleopolyhedrovirus	ThorNPV	NC_019945
<i>Trichoplusia ni</i> single nucleopolyhedrovirus	TniSNPV	NC_007383
Betabaculovirus		
<i>Adoxophyes orana</i> granulovirus	AdorGV	NC_005038
<i>Agrotis segetum</i> granulovirus	AgseGV	NC_005839
<i>Choristoneura occidentalis</i> granulovirus	ChocGV	NC_008168
<i>Closteria anachoreta</i> granulovirus	CaGV	NC_015398
<i>Closteria anastomosis</i> granulovirus Henan	ClanGV	NC_022646
<i>Cnaphalocrocis medinalis</i> granulovirus	CnmeGV	NC_029304
<i>Cryptophlebia leucotreta</i> granulovirus	CrleGV	NC_005068
<i>Cydia pomonella</i> granulovirus	CpGV	NC_002816
<i>Diatraea saccharalis</i> granulovirus	DisaGV	NC_028491
<i>Epinotia aporema</i> granulovirus	EpapGV	NC_018875
<i>Erinnyis ello</i> granulovirus	ErelGV	NC_025257
<i>Helicoverpa armigera</i> granulovirus	HearGV	NC_010240
<i>Mocis</i> sp. granulovirus	MospGV	NC_029996
<i>Mythimna unipuncta</i> granulovirus	MyunGV	NC_033780
<i>Phthorimaea operculella</i> granulovirus	PhopGV	NC_004062
<i>Pieris rapae</i> granulovirus	PrGV	NC_013797
<i>Plodia interpunctella</i> granulovirus	PiGV	NC_032255
<i>Plutella xylostella</i> granulovirus	PlxyGV	NC_002593
<i>Pseudaletia unipuncta</i> granulovirus	PsunGV	NC_013772
<i>Spodoptera frugiperda</i> granulovirus	SfNPV	NC_026511
<i>Spodoptera litura</i> granulovirus	SpliGV	NC_009503
<i>Xestia c-nigrum</i> granulovirus	XecnGV	NC_002331
Gammabaculovirus		
<i>Neodiprion abietis</i> NPV	NeabNPV	NC_008252
<i>Neodiprion lecontei</i> nucleopolyhedrovirus	NeleNPV	NC_005906
<i>Neodiprion sertifer</i> nucleopolyhedrovirus	NeseNPV	NC_005905
Deltabaculovirus		
<i>Culex nigripalpus</i> nucleopolyhedrovirus	CuniNPV	NC_003084
Unclassified Baculoviridae		
<i>Perigonia lusca</i> single nucleopolyhedrovirus	PeluSNPV	NC_027923
<i>Urbanus proteus</i> nucleopolyhedrovirus	UrprNPV	NC_029997

*List adapted from National Center for Biotechnology information (NCBI) Entrez Genome (November 2017)

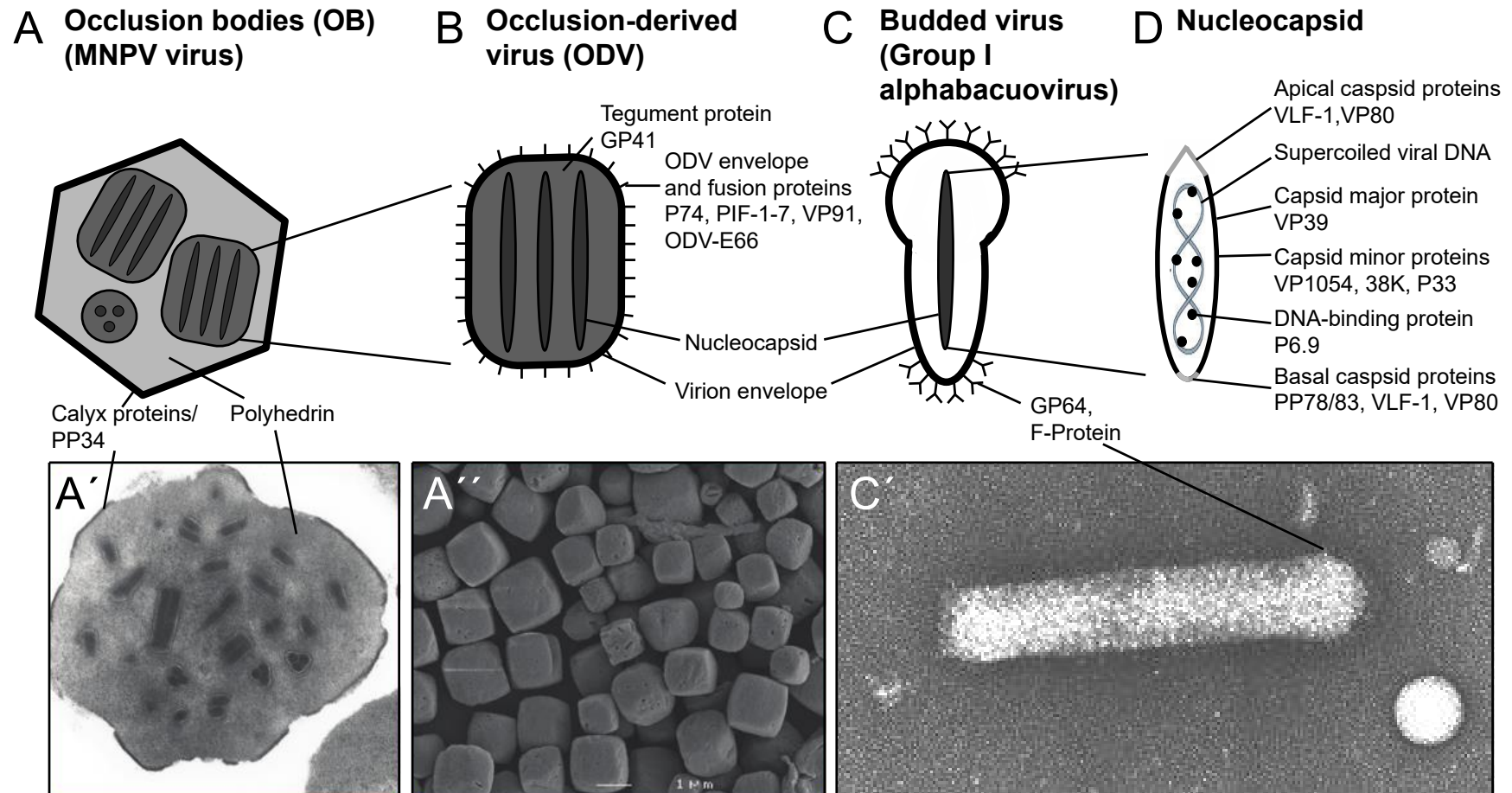


Figure 1.1 Morphology of OBs, ODVs and BVs of Group I alphabaculoviruses

A) Schematics of occlusion bodies (OB) accommodating multiple occlusion-derived virions (MNPV- multiple nucleopolyhedrovirus). OBs contain a crystalline matrix consisting of polyhedrin protecting the virions from some environmental stress factors and embedding the occlusion-derived viruses (ODVs). OBs are surrounded by an envelope, which is mainly composed of the viral PP34. On the contrary to OBs, B) ODVs are highly alkali resistant and mediate primary infection in the midgut of infected insects via the so-called *per os* infectivity factors (PIFs) and other ODV envelope proteins. C) Secondary infection *in vivo* is mediated by budded viruses (BVs). Both ODVs and BVs are genetically identical. However, they differ in the composition of their structural and envelope protein as well as in the number of nucleocapsids they contain per virion. Usually, BVs contain one nucleocapsid per envelope, whereas ODVs hold a bundle of nucleocapsids. The BVs envelope consists of the envelope proteins glycoprotein 64 (GP64) and fusion (F-) protein. D) The viral nucleocapsid is composed of the viral major capsid protein viral structural protein (VP39) and minor casid proteins such as the very late factor (VLF-1), VP80, 38K, P33 and PP78/83. The nucleocapsid contains the supercoiled viral DNA, which is probably condensed by the viral protein P6.9. A') Transmission electron microscopy and A'') Scanning electron microscopy of OBs. C') Electron micrograph from BV. (Images A' was provided by Insect Virus Research Group (IVRG), Oxford Brookes University, A'' was taken by S. Irons, both with permission, C' was taken from "The Big Picture Book at viruses" by Eric B. Carstens, Queens University, Ontario, Canada, 1996, with permission)

1.4 Structure of BVs and ODVs

A prominent feature of baculoviruses and therefore for AcMNPV, is the production of two progeny forms of virions. The BV is released from the plasma membrane, whereas the other form becomes enveloped and occluded (occlusion-derived virus, ODV) into OBs inside the nucleus, which are released upon cell lysis. Although BVs and ODVs are genetically identical, they contrast morphologically and biophysically in the number of nucleocapsids they contain per virus particle. Their envelope and protein composition also differs (Fig. 1.1; Summers and Volkman, 1976; Volkman *et al.*, 1976; Pearson *et al.*, 1988).

Baculovirus BVs were originally described as rod-shaped (Fig. 1.1C; Beaton and Filshie, 1976; Fraser, 1986) although recent investigation by cryo-electron microscopy (EM) revealed an elongated, ovoid shape with a mostly apical distribution of envelope proteins. However, a few envelope proteins were found on lateral regions (Wang *et al.*, 2016). The AcMNPV envelope fusion protein GP64, which is one of the three most abundant proteins associated with BV, is a fatty acid acylated glycoprotein composed of trimers (Roberts and Faulkner, 1989; Wang *et al.*, 2010b, 2016). Two trimers assemble into spikes on the envelope surface (reviewed by White *et al.*, 2008). The BV envelope consists of a ~6-7 nm thick bilayer deriving from the plasma membrane of their host (Braunagel and Summers, 1994; Wang *et al.*, 2016). Usually, BVs contain one nucleocapsid per envelope, which possibly interacts with the viral envelope at the apical poles (Wang *et al.*, 2016).

On the contrary, a bundle of viral nucleocapsids is enveloped into an ODV derived from the nuclear membrane (Fig. 1.1B; Stoltz *et al.*, 1973; Shi *et al.*, 2015). The ODV envelope consists of the conserved *per os* infectivity factors (PIFs), which form complexes on the ODV envelope surface mediating viral entry into the insect midgut epithelial cells (reviewed by Wang *et al.*, 2017). So far, nine PIFs have been described: P74 (PIF0; Kuzio *et al.*, 1989; Faulkner *et al.*, 1997), PIF-1 (Kikhno *et al.*, 2002), PIF-2 (Pijlman *et al.*, 2003), PIF-3 (Ohkawa *et al.*, 2005), PIF-4 (Fang *et al.*, 2009a), PIF-5 (Sparks *et al.*, 2011), PIF-6 (Nie *et al.*, 2011), Ac110/PIF-7 (Liu *et al.*, 2016) and VP91 (Zhu *et al.*, 2013; Javed *et al.*, 2016). Deletion of any of the PIFs causes loss of oral infectivity in insects but does not affect BV production with the exception of VP91 (reviewed by Wang *et al.*, 2017). The presence of a tenth PIF (Ac83/P96) has been proposed (Javed *et al.*, 2017).

1.4.1 Virus capsid and VP39

The viral capsid forms spiral structures of ~310 nm in length and ~50 nm in diameter with distinct polar ends (Fig. 1.1D; Wang *et al.*, 2016). Inside the viral nucleocapsids, the small basic (arginine-rich) viral DNA-binding protein P6.9 probably condenses the viral DNA,

which is possibly regulated by phosphorylation of P6.9 (Tweeten *et al.*, 1980; Wilson *et al.*, 1987; Wang *et al.*, 2010a).

The major component of the capsid is the virus structural protein 39 (VP39; Thiem and Miller, 1989b), which is one of the three most abundant proteins in BVs (Wang *et al.*, 2010b). Minor capsid proteins include the viral very late factor 1 (VLF 1; Yang and Miller, 1998), GP41 (Whitford and Faulkner, 1992), P33 (Wu and Passarelli, 2010; Nie *et al.*, 2011), VP80 (Lu and Carstens, 1992; Braunagel *et al.*, 2003) and PP78/83 (Fig. 1.1D; Vialard and Richardson, 1993; Russell *et al.*, 1997).

The VP39 was first characterised in OpMNPV as the major viral capsid protein that migrates at 39 kDa during sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of virus-infected cell lysates (Pearson *et al.*, 1988). Subsequently, VP39 was identified as a viral late protein in AcMNPV (late gene expression: Introduction 1.5.4), which is maximally transcribed from 12-24 hours post infection (h p.i.; Thiem and Miller, 1989b).

Immunofluorescence studies revealed that VP39 co-localised with both the viral ME53 and GP64 at distinct areas of the cell plasma membrane, which possibly represent sites for virion budding. Furthermore, ME53 was associated with the capsids in BVs. It was therefore hypothesised that ME53 acts as a scaffold protein connecting the viral nucleocapsid with its envelope in BVs (de Jong *et al.*, 2011).

The ODVs are enclosed by the crystalline matrix protein polyhedrin, which in turn is surrounded by an electron-dense calyx/polyhedron envelope (PE) with the viral protein PP34 as a major component (Fig. 1.1A; Whitt and Manning, 1988). Although BVs and ODVs differ in their structures, they contain the same nucleocapsids.

Deletion of the *vp39* coding sequence from the alphabaculovirus *Bombyx mori* NPV (BmNPV) failed to produce apparent progeny viruses but did express *polh* (Ono *et al.*, 2012). Moreover, the highly conserved glycine (Gly, G) 276 in BmNPV VP39 has been implicated to be crucial for proper VP39 function including nucleocapsid assembly, viral DNA packaging but also viral late gene expression (late gene expression: Introduction 1.5.4) by random mutagenesis studies with 5'-bromdeoxyuridine (BrdU). The synthetic nucleoside BrdU is an analogue of thymine and can base pair with guanine residues thereby inducing point mutations during viral propagation. After BrdU mutagenesis a BmNPV was isolated containing a few polyhedra phenotype (FP). This virus isolate accommodated a point mutation in VP39 from Gly 276 to serine (Ser, S). Cells infected with this mutant virus had a reduced expression of the viral late gene *cathepsin* (*cath*: Introduction 1.6.1) and the viral very late genes (viral very late gene expression:

Introduction 1.5.6) *p10* and *polh* as examined by real time polymerase chain reaction (RT-PCR; Katsuma and Kokusho, 2017). This indicated that VP39 might have additional functions during viral gene expression apart from acting as a structural protein.

However, in order to generate nucleocapsid and progeny viruses, baculoviruses need to replicate their genome inside their host cell.

1.5 The AcMNPV replication cycle

The baculovirus and, therefore the AcMNPV, replication cycle can be divided into four distinct phases: The immediate early, delayed early, late and very late phase of infection (Fig. 1.2). The early phase is characterised by the transcription of viral genes required for viral replication and for the establishment of an infection. Whereas immediate early genes initiate the viral transcription cascade independently of other viral gene products, delayed early genes are expressed slightly later (Guarino and Summers, 1986, 1987, Carson *et al.*, 1988, 1991a). The late phase of infection is characterised by a switch from the host RNA polymerase to the virus encoded α -amanitin resistant RNA polymerase (Fuchs *et al.*, 1983; Guarino *et al.*, 1998). In this phase of infection BVs are mainly being produced and released. Subsequently, in the very late phase the virions are enveloped within the nucleus and occluded into polyhedra. Furthermore, in the very late phase the viral genes *polh* and *p10* are hyperexpressed (Smith *et al.*, 1983a; Pennock *et al.*, 1984; Vlak *et al.*, 1988). Typically, early and late viral genes are characterised based on their promoter motifs.

1.5.1 AcMNPV promoter motifs

Baculovirus early promoter motifs are similar to eukaryotic promoter structures. Important for their regulation is the binding of and activation by host transcription factors (Hoopes and Rohrmann, 1991; Kogan and Blissard, 1994). Usually, they contain the conserved eukaryotic transcription initiation and binding site CAGT that is located downstream of the eukaryotic TATA box motif (Blissard and Rohrmann, 1989). Deletion and substitution studies of early promoter motifs revealed that early transcription is mediated by a minimum promoter consisting of the TATA element. However, the CAGT motif is required for full promoter activity. Deletion of CAGT resulted in the reduction of promoter gene activity and *in vitro* transcripts although these are still accurately initiated (Dickson and Friesen, 1991; Blissard *et al.*, 1992). Transcript levels of viral genes transcribed from early promoter motifs are usually highest between 6-12 h p.i. (Chen *et al.*, 2013).

The viral late promoter consists of the transcription start site (TSS) TAAG, from which transcription is specifically initiated at the second nucleotide by the viral RNA polymerase (Kuzio *et al.*, 1984; Rankin *et al.*, 1988). Transcription of viral genes from late promoter

motifs is typically highest between 12-18 h p.i. or later (Chen *et al.*, 2013). Interestingly, the strength of late gene expression seems to be determined by the presence of the immediate context of the TAAG sequence (Ooi *et al.*, 1989; Morris and Miller, 1994).

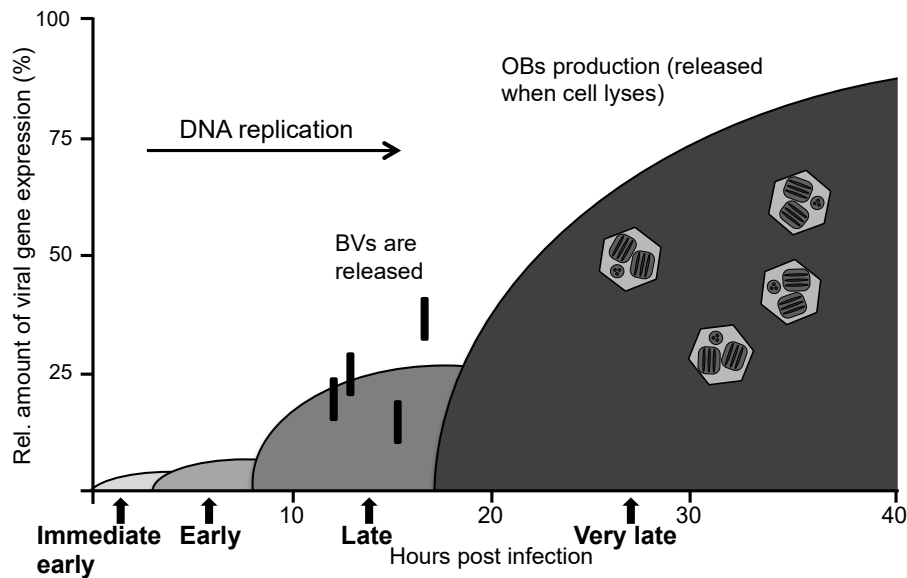


Figure 1.2 Theoretical model of AcMNPV gene expression

AcMNPV gene expression is highly regulated and genes are expressed in early, late and very late phases. During the early events, the viral infection is established and viral genes are transcribed for replication. In the late phase of infection budded viruses (BV) are produced, whereas polyhedra (OB: occlusion body) are assembled and released within the nucleus in the very late phase of infection. The very late phase terminates with cell lysis.

Both *polh* and *p10* contain an AT-rich sequence referred to as the “burst-sequence” downstream of their late promoter sequence, which is essential for their high levels of expression. Deletion studies with the progressive removal of nucleotides upstream of the ATG translational start codon using an *in vitro* transient expression assays indicated that the entire 5′ non-coding (leader) sequence is necessary for high levels of gene expression (Matsuura *et al.*, 1987; Weyer and Possee, 1988). On the contrary, a similar approach revealed that only a minimal nucleotide sequence upstream from the messenger (m)RNA start site (TAAG) is required for effective very late gene expression. Only 69 nucleotides (nt) and 101 nt upstream of the *polh* or *p10* ATG codon is sufficient for obtaining high levels of promoter activity in transient expression systems. The removal of a TATA-like motif did not affect the levels of very late gene expression (Possee and Howard, 1987; Weyer and Possee, 1989). Furthermore, it seems that expressional competition between the *polh* and *p10* promoters occurs. Deletion of the *p10* promoter increased gene expression from the *polh* promoter (Chaabihi *et al.*, 1993). This might lead to the impression that *p10* and *polh* promoter activity are regulated the same. However, expression from *p10* occurs several hours earlier than from *polh* (Smith *et al.*, 1983c; Roelvink *et al.*, 1992) and moreover, deletion of the *polh* promoter sequence did not affect *p10* expression (Chaabihi *et al.*, 1993).

A global analysis of TSS present in AcMNPV-infected *T. ni* cells by 5' rapid amplification of complementary (c)DNA-ends with polymerase chain reaction (RACE) Illumina® sequencing revealed a total number of 218 TSS that were associated with 156 viral orfs. Of those, 92 TSS did not initiate at TAAG motifs and therefore, they likely represent early transcripts. These 92 TSS were associated with 77 orfs and only 29 of these contained a TATA box motif. The majority of viral transcripts (126 TSS) initiated at a TAAG motif, which typically presents late transcription. The 126 TSS could be found associated with 101 orfs and 80 of these orfs contained only one mapped late promoter, whereas 21 orfs had two or more late promoter motifs. Based on this, it was estimated that the AcMNPV genome consists of 34.6% early, 51.9% late and 13.5% early and late genes (Chen *et al.*, 2013).

1.5.2 Early phase of viral infection

However, before any of the viral genes can be expressed, baculoviruses need to be internalised by their host cells.

In vivo, baculoviruses are taken up by the insects *per os* (Fig. 1.3). Upon exposure to the high pH conditions in the insect midgut (pH 8.5-11), the alkali-sensitive polyhedra dissolve, thereby releasing ODVs. The ODVs are highly alkali-resistant and are able to initiate primary infection in the midgut epithelial cells, whereby infection of both columnar epithelial and regenerative cells of the insect midgut seems to occur equally. The initiation of infection through the midgut peritrophic matrix is most likely facilitated by viral PIFs and some other ODV envelope proteins such as ODV-E66 (Keddie *et al.*, 1989; Faulkner *et al.*, 1997; Sugiura *et al.*, 2011).

Conversely, the establishment of a secondary infection inside the insects requires BVs. The BVs bud through the basolateral membrane of infected midgut cells entering the hemocoel from which they cause systemic infections in susceptible tissues and organs such as the tracheal epithelium but also the hemocytes and the fat body (Fig. 1.3; Keddie and Volkman, 1985; Keddie *et al.*, 1989).

Binding of BVs from group I NPVs is mediated by the viral glycoprotein GP64 via an unknown receptor and the potential involvement of phospholipids. Virion entry occurs into clathrin-coated pits via the cellular endosomal pathway (Blissard and Wenz, 1992; Monsma *et al.*, 1996; Tani *et al.*, 2001; Long *et al.*, 2006). Once taken up into the cytosol, it has been suggested that the viral protein PP78/83 acts as an actin nucleating promoting factor for the retrograde trafficking of nucleocapsids towards the cell nucleus where they traverse the nuclear membrane through the nuclear pores (Goley *et al.*, 2006; Ohkawa *et al.*, 2010). After the viral DNA is uncoated and released inside the nucleus, expression of

the highly temporal regulated viral genes occurs (Kelly and Lescott, 1981; Friesen and Miller, 1985).

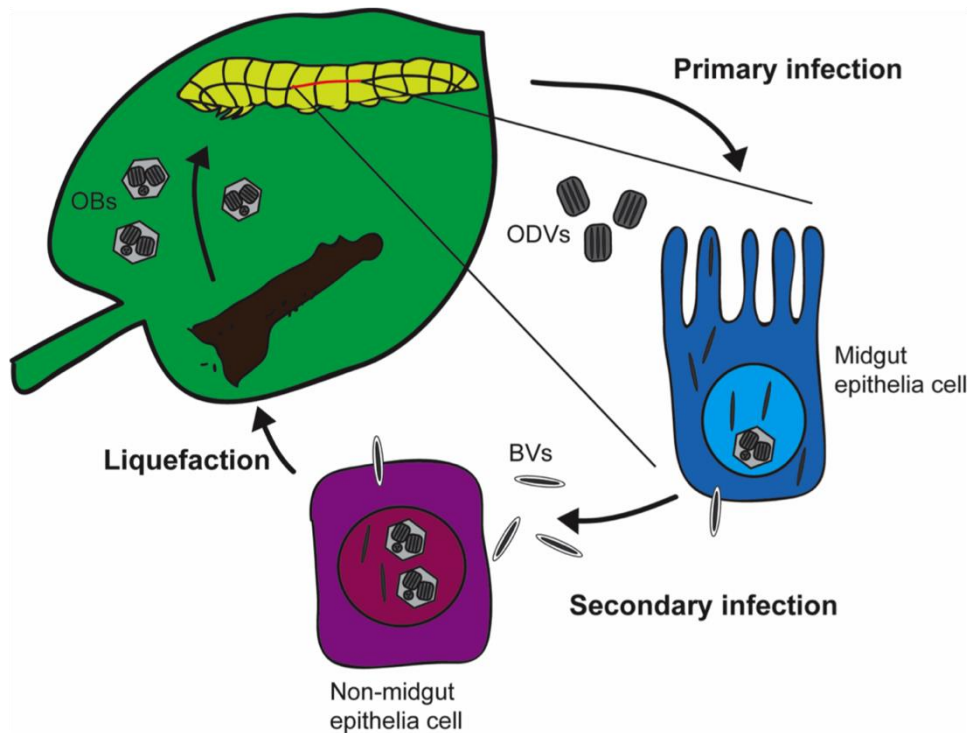


Figure 1.3 Baculovirus infection *in vivo*

Baculoviruses are taken up as occlusion bodies (OBs) by the insect with their food. Once the OBs enter the highly alkali environment of the insect midgut, they are dissolved thereby releasing the occlusion-derived virions (ODVs), which mediate primary infection in the midgut epithelial cells. Secondary infection inside susceptible insect tissues is mediated by budded viruses (BVs). At the end of the viral replication cycle, OBs are released upon host cell lysis and liquefaction of the insect.

Infection is associated with cell cycle arrest, shut-down of host gene expression and the formation of an electron-dense, chromatin-like structure inside the nucleus, the so called virogenic stroma. The virogenic stroma is the location of viral replication and related to the packaging of the viral DNA and assembly of the nucleocapsids (Fraser, 1986).

1.5.3 Viral replication

The mechanism by which AcMNPV replicates its genome is still controversial. Originally, it was suggested that the viral DNA might be replicated by a rolling circle mechanism (Oppenheimer and Volkman, 1997). Alternatively, there was evidence for a recombinant-dependent replication (Martin and Weber, 1997). More recently, it has been hypothesised that the viral genome might be replicated by a combination of both a rolling circle and recombinant-dependent replication (reviewed by Rohrmann, 2013).

Viral DNA synthesis begins as early as 4 h p.i. and originates at six homologous regions (hr; Pearson *et al.*, 1992). However, viral replication probably does not initiate exclusively

at *hr* regions (Habib and Hasnain, 2000).

Viral genes required for AcMNPV DNA synthesis were identified by a transient *in vitro* replication assay. An AcMNPV genome library was transfected along with a plasmid containing an AcMNPV DNA replication origin. Viral replication was measured by a *DpnI*-sensitive assay to distinguish between input and newly replicated viral DNA. This method identified six viral genes to be essential for viral DNA replication: *DNA polymerase* (*dnapol*), *immediate-early protein 1* (*ie-1*), *p143*, *late expression factor 1* (*lef-1*), *lef-2* and *lef-3*. Three genes encoding for P35, IE-2 and PE38 were determined to have a stimulatory effect on DNA replication (Fig. 1.4; Table 1.2; Kool *et al.*, 1994).

Table 1.2 Viral genes required for replication as identified by transient expression assays*

Gene name	Protein function	Reference
<i>ie-1</i>	Early gene transactivator	(Guarino and Summers, 1986)
<i>lef-1</i>	DNA primase	(Mikhailov and Rohrmann, 2002)
<i>lef-2</i>	DNA primase accessory protein	(Mikhailov and Rohrmann, 2002)
<i>lef-3</i>	ssDNA binding protein	(Hang <i>et al.</i> , 1995)
<i>dnapol</i>	DNA polymerase	(Tomalski <i>et al.</i> , 1988)
<i>p143</i>	DNA helicase	(McDougall and Guarino, 2000)
<i>p35</i>	Anti-apoptotic protein	(Clem <i>et al.</i> , 1991)
<i>ie-2</i>	Early gene transactivator	(Carson <i>et al.</i> , 1988)
<i>pe38</i>	Transcriptional regulator	(Krappa and Knebel-Mörsdorf, 1991)

*Viral genes found to be essential for viral replication are shown in bold, whereas the other three viral genes only had stimulatory effect on viral replication (Kool *et al.*, 1994; Lu and Miller, 1995)

However, there has been some controversy as to whether *dnapol* is essential for viral replication. An experimental approach similar to that described above suggested that *dnapol* had only stimulatory effects on viral replication (Lu and Miller, 1995). During transient expression assays, the expression vector containing a reporter gene under the viral promoter is provided and may not reflect the same activity as in a physiological context. Hence, results obtained from transient expression assays need confirmation and validation, e.g. by knock-down or knock-out experiments. When AcMNPV *dnapol* was knocked down by dsRNA, viral replication did not occur (Schultz and Friesen, 2009). Additionally, *dnapol* knock-out AcMNPV was unable to replicate viral DNA when transfected in insect cells and the host DNAPol could not substitute for this effect. Viral DNA was replicated, when cells were infected with a rescue virus (Vanarsdall *et al.*, 2005). Moreover, *dnapol* seems to be essential in BmNPV (Gomi *et al.*, 1997; Ono *et al.*, 2012). Overall, these results indicate that AcMNPV DNAPol might be crucial for viral replication.

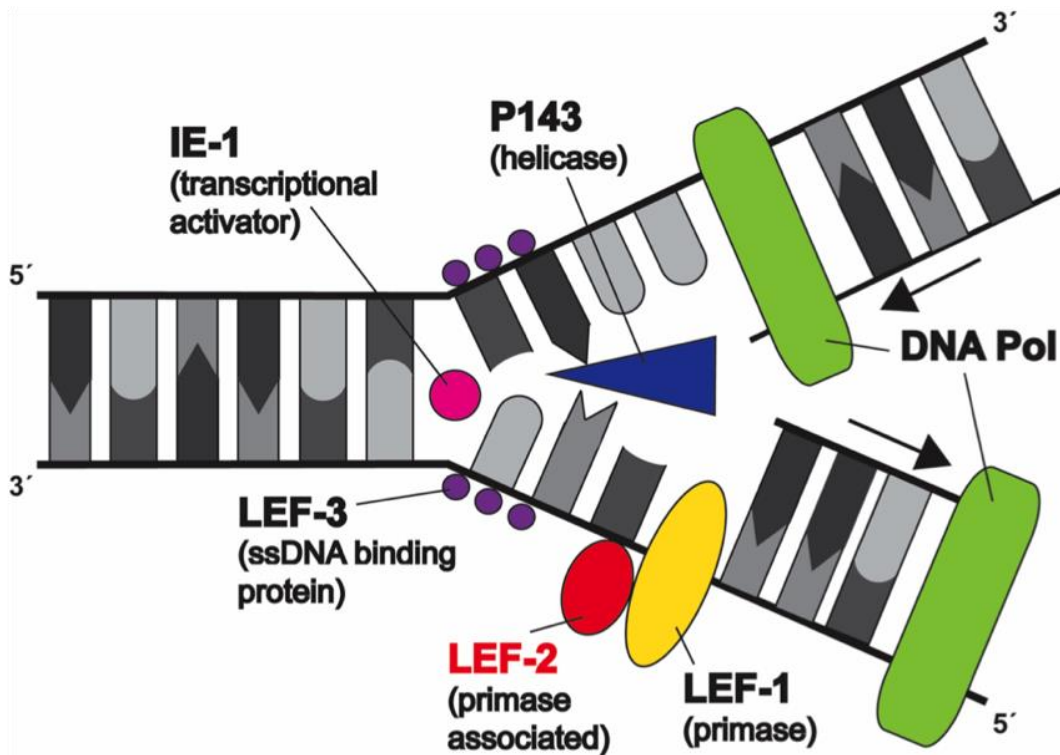


Figure 1.4 Virus DNA replication

Potential model of how AcMNPV DNA is replicated in insect cells. The transcriptional activator IE-1 recruits the helicase P143 and the single-stranded (ss)DNA binding protein LEF-3. LEF-3 maintains the DNA strands separated. The primase LEF-1, which is incorporated into the replication complex by the primase associated protein LEF-2, synthesizes RNA primers as a starting point for the DNA synthesis. DNA synthesis is mediated by the DNA Polymerase (DNAPol).

During viral replication, the transcriptional activator IE-1 binds to *hr* sequences within the AcMNPV genome. Phosphorylation of the amino- (N-) terminal replication domain (TPXR/H) of IE-1 probably coordinates viral DNA replication by the initiation of a replication complex with the P143 and LEF-3 (Fig. 1.4; Taggart *et al.*, 2012). Recruitment of the DNA helicase P143 results in unwinding of the two annealed DNA strands and the generation of a replication fork (McDougal and Guarino, 2000). The DNA strands are kept separated by LEF-3, which is a single-stranded (ss) DNA binding protein (Hang *et al.*, 1995). The viral LEF-2 acts as a DNA primase accessory protein able to bind both LEF-1 and DNA, and possibly incorporating LEF-1 into the replication complex on the viral DNA. After the DNA strands are separated, LEF-1 acts as a DNA primase and catalyses short RNA sequences (primers), which serve as starting points for DNA synthesis for DNAPol (Mikhailov and Rohrmann, 2002). The highly conserved DNAPol contains 3'→5' exonuclease activity specific for ssDNA. Therefore, it has been suggested that it also contains proof-reading activity (Mikhailov *et al.*, 1986; Tomalski *et al.*, 1988; McDougal and Guarino, 1999). A DNA ligase that joins the Okazaki fragments after removal of the RNA primers at the lagging strand has not been identified in alphabaculoviruses yet (Mikhailov and Rohrmann, 2009). However, a DNA ligase

characterised in gammabauloviruses was found to be nonessential for replication. Therefore, it was hypothesised that the host ligase undertakes this function (Fig. 1.4; Pearson and Rohrmann, 1998).

The virus genes identified that only had stimulatory effects on replication seem to play an indirect role during this process. Viral gene *ie-2* transactivates early gene expression and blocks cell cycle progression (Carson *et al.*, 1988; Prikhod'ko and Miller, 1998; Prikhod'ko *et al.*, 1999), whereas the P35 acts as an anti-apoptotic protein (Clem *et al.*, 1991). The P35 is a cell line specific protein blocking the apoptotic response in virus-infected cells (Clem *et al.*, 1991; Hershberger *et al.*, 1992; Gross and Shuman, 1998; Griffiths *et al.*, 1999). Deletion of the very early gene *pe38* significantly delayed viral DNA synthesis by ~3 h and reduced BV production by more than 99% *in vitro* when compared to wild-type (wt)-infected cells. Moreover, the same mutant impacted viral virulence and reduced oral infectivity in bioassays (Milks *et al.*, 2003). The examination of the viral transcription cascade in Ac Δ pe38 infected cells by time course microarray studies further revealed that *pe38* seemed to be crucial for the transactivation of five genes: *lef-3*, *dnapol*, *he65*, *p143*, which in turn affected the transcription of *p35* and *gp64* (Jiang *et al.*, 2006).

Three other viral *lefs*: *lef-10*, *lef-11* and *lef-7*, which were not identified during transient replication assays, have been implicated to play a role during viral genome replication:

Knock-out of the viral *lef-10* in both AcMNPV and BmNPV abolished the production of viral progeny in transfected insect cells although a repair virus could rescue BV production (Yu *et al.*, 2013; Xu *et al.*, 2016).

Analysis of viruses where *lef-11* had been deleted were unable to propagate *in vitro*. Southern dot blot hybridisation revealed that the genome of this mutant was not amplified and Northern blot analysis for selected viral genes suggested that viral early, late and very late transcripts were absent (Lin and Blissard, 2002). Moreover, BmNPV LEF-11 did not only interact but could also directly activate and regulate host ATPase family members heat shock protein family D (Hsp60) member 1 and ATPase family, AAA domain 3A (ATAD3A), which both contributed to viral replication (Dong *et al.*, 2017).

Deletion of *lef-7* only delayed DNA synthesis after AcMNPV infection of the permissive cell line *Trichoplusia ni* 368 (TN368) for 12-18 h, whereas it was reduced to 5% in the non-permissive insect cell line *Spodoptera frugiperda* 21 (Sf21) (Chen and Thiem, 1997). Although AcMNPV was isolated from a different host than TnMNPV, both virus isolates are genetically almost identical. Hence, *T. ni* can be considered as the natural host for AcMNPV (Smith and Summers, 1979). The *lef-7* gene product contains a highly conserved nuclear F-box motif, which mediates protein-protein interactions with the host

synthesis (S)-phase kinase associated protein (SKP1). Moreover, it suppresses host DNA-damage-induced accumulation of the phosphorylated host histone variant H2AX. Deletion of the N-terminal F-box motif resulted in H2AX accumulation during viral DNA synthesis and reduced both the production of infectious progeny as well as viral very late gene expression (Mitchell *et al.*, 2013).

In addition, the cellular phosphoinositide-protein kinase B (PI3K-Akt) signalling pathway was suggested to play an important role for efficient viral replication. Inhibition of PI3K reduced both BV and ODV production. Furthermore, inhibition had no effect on viral early gene (*ie-1*) and early-late gene (*gp64*) expression, whereas *vp39* and *polh* expression and viral DNA replication were decreased (Xiao *et al.*, 2009).

Following viral DNA replication, VLF-1 may be required for the DNA packing process and nucleocapsid assembly inside the virogenic stroma (Vanarsdall *et al.*, 2005, 2006).

1.5.4 Late phase of infection

The late phase of viral infection is characterised by a switch from the host RNA polymerase to the virus encoded α -amanitin resistant RNA polymerase (Fuchs *et al.*, 1983; Guarino *et al.*, 1998). The viral RNA polymerase recognises unique promoter motifs (TAAG) from which the viral mRNA is initiated (Introduction 1.5.1; Rankin *et al.*, 1988; Chen *et al.*, 2013).

During the late phase of virus infection, the assembled nucleocapsids egress the nucleus. For this, several mechanisms have been proposed: via the nuclear pore complexes, the nuclear membrane or the endosomal system (Fig. 1.5A; Hess and Falcon, 1978; Fraser and Hink, 1982; Williams and Faulkner, 1997; Au and Panté, 2012). Virions are most likely transported by microtubules to the cell surface, which is facilitated via kinesin-1 interaction with VP39 (Volkman and Zaal, 1990; Fang *et al.*, 2009b; Danquah *et al.*, 2012), and BVs are released from the GP64-enriched plasma membrane from which they obtain their envelope (Oomens and Blissard, 1999).

1.5.5 Viral Late Expression Factors (LEFs)

a. LEF-2

One LEF that has been studied in more detail and has been implicated to be required in several steps of the viral replication cycle is LEF-2.

Historically, the genome organisation of AcMNPV has been divided into fractions produced by restriction endonucleases. Sequencing of the 9.4 kbp *EcoR*-I and –R fragment originally identified the viral orf Ac6 that was later renamed *lef-2*. This 633 bp long core gene encodes for 210 amino acids with a predicted size of 23.9 kilo Dalton

(kDa; Possee *et al.*, 1991; Ayres *et al.*, 1994).

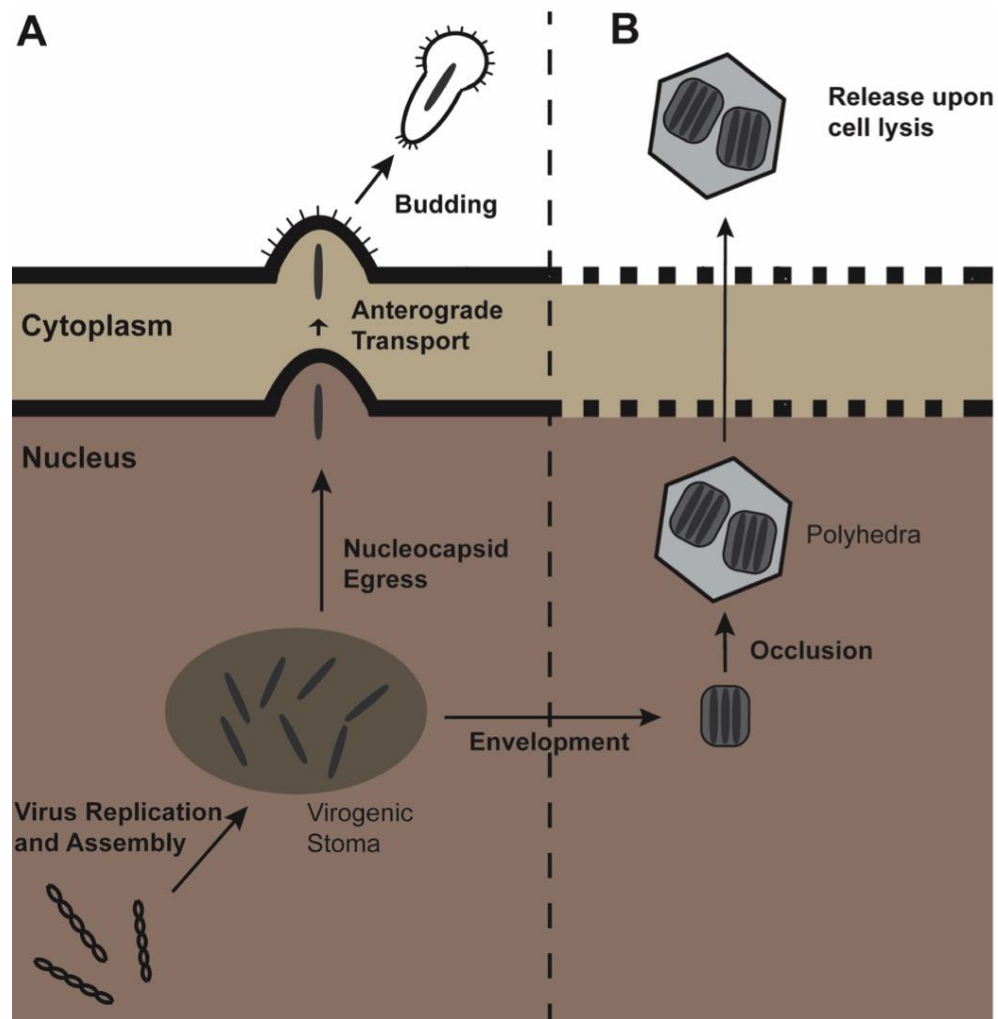


Figure 1.5 The late and very late phase of viral infection

After viral replication, the nucleocapsids assemble inside the nucleus in an electron dense space the so-called virogenic stoma. A) In the late phase of viral infection the nucleocapsids egress the nucleus via an unknown mechanism, transiently obtaining a membrane, which is subsequently lost. Transport towards the plasma membrane occurs in a microtubule and kinesin-dependent manner (anterograde transport). The nucleocapsids bud from the membrane, which is already enriched in the viral glycoprotein GP64. B) In the very late phase of infection the nucleocapsids are enveloped and become occluded into polyhedra inside the nucleus. At the very end of the viral replication cycle the cell lyses releasing the polyhedra.

The *lef-2* mRNA is expressed throughout the complete course of the AcMNPV replication cycle and appears to be one of the most abundant expressed viral genes at 6, 24, 36 and 48 h p.i. (Chen *et al.*, 2013). It is expressed from both an early promoter and a TAAG late promoter motif. Its 5'TSS were mapped to positions: -38 nt (early) and -58 nt (late) upstream of the *orf* start codon. The 3'polyadenylation signal (PAS) AAUAAA was determined to be located 23 nt downstream of the stop codon. Mapping was performed by RACE sequencing (Chen *et al.*, 2013). The temporal expression profiles of BmNPV *lef-2* (210 amino acids, 96% amino acid and 95% protein sequence identity to AcMNPV) also revealed the presence of two transcripts, an early and a late, whereas the early BmNPV

transcript originates -35 nt downstream to the TATA box (Sriram and Gopinathan, 1998). Moreover, the transcriptional mapping of the baculovirus OpMNPV *lef-2* (204 amino acids, 54.7% amino acid sequence identity to AcMNPV *lef-2*) identified both an early and late *lef-2* transcript. The OpMNPV early transcript starts at -30 nt downstream of the late TATA promoter motif (Ahrens and Rohrmann, 1995).

The temporal distribution of AcMNPV LEF-2 was analysed by a *lef-2* repair bacmid (bacmid: Introduction 1.7) containing the *lef-2* coding sequence 5' HA-tagged under its own promoter along with *enhanced green fluorescent protein (egfp)* replacing *polh*. Immunofluorescence analysis as well as cell fractionation studies with antibodies against HA indicated that LEF-2 localises in both the nucleus and cytoplasm of virus-infected cells. Localisation of LEF-2 in the cytoplasm was detected from 16 h p.i., and from 20 h p.i. in the nucleus, respectively. Moreover, LEF-2 might be a component of the viral nucleocapsid in both BVs and ODVs (Wu *et al.*, 2010).

On the contrary, *lef-2* that was *c-myc* tagged at its 5' end in its natural locus was detected already at 4 h p.i. as ~25 kDa protein by immunoblotting of virus-infected cell lysates. It localised mainly in the nucleus both early (9 h p.i.) and late (24 h p.i.) in virus infected cells (R. D. Possee, personal communication).

Transient replication assays in *Sf21* cells identified *lef-2* as a gene essential for viral DNA replication in AcMNPV (Introduction 1.5.3; Kool *et al.*, 1994). With a similar assay, *lef-2* was identified to play an essential role for OpMNPV replication (Ahrens and Rohrmann, 1995). However, when an AcMNPV *lef-2* knock-out virus was transfected, viral replication could be detected by a *DpnI* sensitive DNA replication assay although it was greatly delayed and decreased. It was hypothesised that LEF-2 may not be required for the initiation of viral replication but rather for the amplification of DNA replication (Wu *et al.*, 2010).

Using the yeast-two-hybrid system and the subsequent confirmation by a glutathione S-transferase (GST) assay, it was determined that LEF-2 interacts with LEF-1. The amino acids sequences involved in this interaction were mapped by *lef-2* deletion mutants. The LEF-1 protein interacting domain in LEF-2 was located between amino acids 20-40 close to the LEF-2 N-terminus, whereas the protein interacting domain in LEF-1 could not be identified to a specific amino acid sequence. It was hypothesised that secondary structures are essential for the interaction (Evans *et al.*, 1997).

Whereas LEF-1 showed primase activity *in vitro* but had a very low affinity for binding ssDNA, LEF-2 was not required for LEF-1 primase activity. However, LEF-2 had a very high affinity for ssDNA. Hence, it may serve as an accessory protein for the stabilisation

and the incorporation of LEF-1 into the viral replication complex (Fig. 1.4; Mikhailov and Rohrmann, 2002).

Transient expression assays (in *Sf21* cells) with a chloramphenicol-acetyltransferase (CAT) reporter gene fused to early (*early-to-late: etl*), late (*vp39*) or very late (*polh*) viral genes and transfection of an overlapping AcMNPV genome library identified *lef-2* to be required for viral late and very late gene expression (Passarelli and Miller, 1993b).

In addition, LEF-2 was suggested to be a potential very late expression factor by random mutagenesis of AcUW1.*lacZ*-infected cells with BrdU (Merrington *et al.*, 1996). In the AcUW1.*lacZ* genome, *lacZ* replaced the *p10* coding sequence. Screening for polyhedrin-negative and colourless plaques in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) after BrdU treatment identified a virus deficient in the regulation of very late gene expression (named: very late deficient 1- VLD1). Complementation assays in combination with sequencing revealed that this deficiency was due to a single amino acid change at position 3620 in the *lef-2* orf (from aspartic acid (D) to asparagine (N): LEF-2^{D178N}). Cells that were further infected with VLD1 replicated viral DNA to a similar amount to AcUW1.*lacZ*-infected cells as indicated by dot blot analysis. Moreover, AcMNPV containing the same point mutation as VLD1 (Ac*lef-2*³⁶²⁰) produced comparable BVs titres as VLD1-infected cells. This suggested a dual role for LEF-2 in both viral replication and viral very late gene expression (Merrington *et al.*, 1996).

Additionally, BmNPV *lef-2* trans-activates viral very late gene expression from both *polh* and *p10* viral promoters in a transient expression assay, when the *luciferase* gene (*luc*) was fused with the viral *polh* promoter. Deletion of *lef-2* from the reporter gene construct reduced *luc* activity by 80% but could be regained after *lef-2* was provided by co-transfection. Similar results were obtained with a *p10* reporter gene construct. Furthermore, when the cysteine (Cys, C)-rich domain at the LEF-2 carboxyl- (C-) terminus was deleted, which resulted in an internal deletion of 96 amino acids including D178 with the last 19 amino acids at the LEF-2 retained, trans-activation from the *polh* promoter did not occur (Sriram and Gopinathan, 1998).

On the contrary, phenotypic grouping of BmNPVs knock-out mutants, in which *egfp* was inserted under the *polh* promoter and the coding region of *lef-2* was deleted, still expressed GFP but did not spread virions, when transfected into Bm5 cells (Ono *et al.*, 2012).

b. Other LEFs

In general, viral *lefs* were identified by transient expression assays, in which overlapping AcMNPV genome libraries were co-transfected along with a reporter gene under a

different viral promoter into *Sf21* cells. All *lefs* supported the expression from viral late but not early promoters. In total, nineteen *lefs* were identified in the AcMNPV genome: *lefs-1-12*, *ie-1*, *ie-2*, *dnapol*, *39K* (*pp31*), *p35*, *p47* and *p143* (Li *et al.*, 1993; Passarelli and Miller, 1993b, 1993a; Lu and Miller, 1995; Todd *et al.*, 1995; Rapp *et al.*, 1998). It was further proposed to consider *ie-0* as a twentieth *lef* as it was able to replace *ie-1* in a transient expression assay (Huijskens *et al.*, 2004). The viral protein IE-0 is a splice product of IE-1 and the only baculovirus protein produced by splicing of mRNA (Kovacs *et al.*, 1991).

A subset of *lefs* is required for optimal viral replication. They are also termed replication *lefs* and include *ie-1*, *ie-2*, *lef-1*, *lef-2*, *lef-3*, *p143*, *dnapol*, *p35*, *lef-7*, *lef-10* and *lef-11* (Introduction 1.5.3; Fig. 1.4; Table 1.2).

The remaining *lefs* are termed transcriptional *lefs* and they are involved in transcription specific activities. Four of the viral LEFs (LEF-4, LEF-8, LEF-9 and p47) are subunits of the viral RNA-polymerase (Guarino *et al.*, 1998), while the precise role for the remaining *lefs* (*lef-5*, *lef-6*, *pp31*) is still unknown (Passarelli and Miller, 1994a; Lin and Blissard, 2002; Yamagishi *et al.*, 2007).

As LEFs play important roles during viral replication and are essential for late gene expression, they present crucial targets for the manipulation of recombinant protein expression in baculovirus expression vectors. It would be especially of interest to know how the single protein LEF-2 is able to be important for various steps for both viral replication and very late gene expression.

1.5.6 Very late phase of infection

In the very late phase of viral infection most of the assembled virions become enveloped and occluded in the nucleus (Fig. 1.5B). A characteristic of this final stage of virus infection is the hyperexpression of the viral genes *p10* and *polh* (Fig. 1.6). Both proteins accumulate to very high levels very late in infection (from ~18 h p.i.), but are dispensable for virus replication *in vitro* (Smith *et al.*, 1983a; Pennock *et al.*, 1984; Vlak *et al.*, 1988). By 24 h p.i. *polh* and *p10* mRNA were the most abundant viral mRNAs and at 48 h p.i. they represented 24% and 7.5% of total mRNA, respectively (Chen *et al.*, 2013).

a. Polyhedrin

Homologs of the structural viral protein polyhedrin (~30 kDa) are found in all members of the family of *baculoviridae* except for *Culex nigripalpus* NPV (CuniNPV; Afonso *et al.*, 2001; Perera *et al.*, 2006). Polyhedrin is a highly symmetric and robust protein that accumulates in the nucleus where it crystallises in a lattice surrounding the virions and offers a degree of protection (Fig. 1.6; Duncan *et al.*, 1983; Ji *et al.*, 2010).

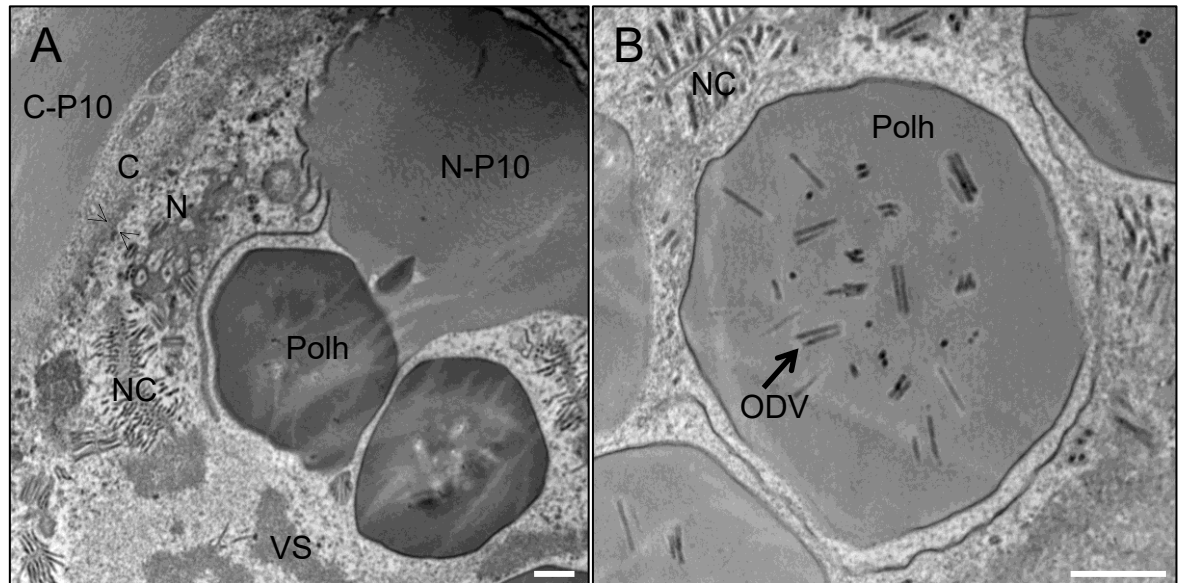


Figure 1.6 P10 and polyhedrin in AcMNPV-infected TN368 cells

A-B) In the very late phase of infection viral *polyhedrin* (*polh*) and *p10* are hyperexpressed. A) P10 forms fibrillar structures in both the cytoplasm (C) and nucleus (N) of infected cells. Polyhedrin accumulates in the nucleus (N), B) where it crystallises around virions embedding the ODVs (occlusion-derived virus). NC: nucleocapsid, VS: virogenic stroma, arrowheads in A) point at nuclear membrane, arrow in B) points at ODV, scale bars are 500 nm (provided by L. P. Graves, with permission).

b. P10

In contrast to polyhedrin, the precise role for the non-structural P10 protein in virus infection is still unclear. It is structurally conserved in all NPVs and most GVs and its amino acid sequence is rich in serine, threonine and proline. The P10 N-terminus consists of a coiled-coil structure and a basic C-terminus, which are separated by a proline-rich sequence (Fig. 1.7; reviewed by van Oers and Vlak, 1997; Carpentier and King, 2009). It appears to have various roles at the end of the virus replication cycle, such as maturation of polyhedra, nuclear stability and microtubule reorganization. However, as its deletion does not affect viral replication it is considered an auxiliary gene (van der Wilk *et al.*, 1987; van Oers *et al.*, 1993; Patmanidi *et al.*, 2003; Carpentier *et al.*, 2008).

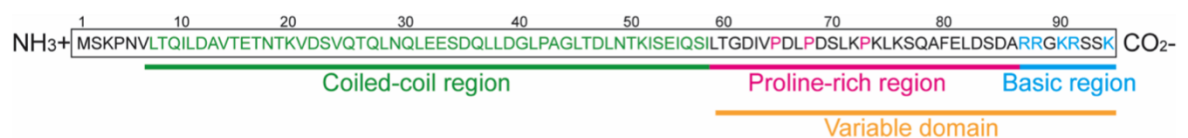


Figure 1.7 Structural organisation of P10

The amino acid sequence of the conserved P10 protein revealed two distinct domains: The coiled-coil domain (green) at the P10 N-terminus and the variable region (orange) at the C-terminus. The variable region is divided into the basis region (blue) containing the negatively charged amino acids arginine (R, in blue) and lysine (K, in blue) and a proline (P)-rich domain (pink).

The *p10* gene product localises in both the cytoplasm and the nucleus of virus-infected

cells (Fig. 1.6A). In the cytoplasm it forms two distinct cytoskeletal-like structures, which do not require any other viral protein for generation (Alaoui-Ismaili and Richardson, 1998; Patmanidi *et al.*, 2003; Carpentier *et al.*, 2008). However, the thin P10 filaments require the integrity of host cell microtubules and furthermore, P10 interacts with host tubulin most likely through its basic region. Hence, depolymerisation of host cell microtubules inhibits P10 filamentous structures (Volkman and Zaal, 1990; Patmanidi *et al.*, 2003). Alternatively, P10 that forms thick rod tubules around the nucleus of virus-infected cells ("perinuclear cage") is not associated with microtubules and is believed to stabilise the nucleus during polyhedra morphogenesis (Carpentier *et al.*, 2008). The structural formation of P10 inside the host cells is moreover regulated by phosphorylation as AcMNPV containing a mutation inside the P10 from serine 93 to alanine (Ala, A) failed to form aberrant perinuclear structures (Raza *et al.*, 2017). Furthermore, both the P10 C- and N-terminus were described to be essential for P10 filament formation (van Oers *et al.*, 1993; Alaoui-Ismaili and Richardson, 1998; Dong *et al.*, 2007).

Additionally, P10 is necessary for OB morphogenesis inside the nucleus by the adhesion of an outer layer of the polyhedra (referred to as an envelope) that appears to stabilise and protect the polyhedra from some physical damage (Vlak *et al.*, 1988; Williams *et al.*, 1989). The deletion of 52 N-terminal amino acids of AcMNPV P10 followed by a *lacZ* insertion either in frame or out of frame prevented the formation of a polyhedra envelope, whereas an AcMNPV that lacked the entire *p10* coding sequence formed fragile polyhedra, in which the envelope was only loosely attached (Williams *et al.*, 1989; van Oers *et al.*, 1993). Furthermore, both mutants failed to lyse and did not release polyhedra as a result of impaired nuclear disintegration (Williams *et al.*, 1989; van Oers *et al.*, 1993). In addition, premature nuclear disintegration could be observed in virus-infected cells, when 50 amino acids of the viral P26 C-terminus were replaced with 43 C-terminal amino acids of P10 in a backbone virus that contained a deletion of the natural *p10*. It was hypothesised that the earlier expression of the P10 C-terminus (as it was expressed under the early *p26* promoter) may account for the premature nuclear lysis (Chaabihi *et al.*, 1993). However, by mutagenesis studies the domain required for nuclear disintegration was mapped to the *p10* *BglII-HindIII* fragment (encoding for P10 amino acids 52-79; van Oers *et al.*, 1993).

c. Other viral genes required for viral very late gene expression

A few viral genes have been already characterised to play an important role for viral very late gene expression. These genes include *fp25k*, *vlf-1*, *pk-1* and *pkip* (Hink and Vail, 1973; Ramoska and Hink, 1974; Ooi *et al.*, 1989; Jarvis *et al.*, 1992; McLachlin and Miller, 1994; Harrison and Summers, 1995; Fan *et al.*, 1996; Harrison *et al.*, 1996; Yang and

Miller, 1998; Mistretta and Guarino, 2005; Mishra *et al.*, 2008; Liang *et al.*, 2017). In addition, the viral LEF-2, which is primarily associated with DNA replication, was identified as a potential candidate for viral very late expression (Introduction 1.5.5a; Merrington *et al.*, 1996).

Gaining a more detailed knowledge about how the very late phase of infection is regulated could be a benefit in the context of baculovirus expression vectors and recombinant protein expression.

1.6 Baculovirus transmission *in vivo*

1.6.1 Cell lysis and release of polyhedra

The very last step in the viral replication cycle is the release of polyhedra, which occurs by cell lysis and nuclear disintegration. *In vivo*, the insect exoskeleton made of chitin has to be disrupted to achieve polyhedra dissemination from the host.

Liquefaction of the insect is mediated by the viral enzymes Cath and Chitinase A (ChitA). Insects infected with AcMNPV either lacking *chitA* or *cath* or both coding regions remained intact even several days after death and did neither become flaccid nor undergo melanisation. Both *cath* and *chitA* genes are auxiliary as they are not required for viral replication (Hawtin *et al.*, 1997). Furthermore, both genes are transcribed as viral late mRNAs from TAAG promoter motifs from 9 h p.i. (Hodgson *et al.*, 2007) and *chitA* is translated from about 12 h p.i. (Thomas *et al.*, 2000; Saville *et al.*, 2004), whereas the translation of *cath* was not detected until 22 h p.i. (Slack *et al.*, 1995). However, different virus strains and cells were utilised for the protein expression studies (Slack *et al.*, 1995; Thomas *et al.*, 2000; Saville *et al.*, 2004).

Moreover, the deletion of both *chitA* and *cath* have been implied to have a positive effect on recombinant protein expression (Introduction 1.7; Kaba *et al.*, 2004; Hitchman *et al.*, 2009).

a. Chitinase A

Chitinases are glycosyl hydrolases degrading the glycosidic bonds in chitin, which is a linear polymer of β -1,4-N-acetylglucosamine (GlcNAc). In general, chitinases are divided into two main groups: Endo- and exochitinases. Whereas exochitinases catalyse the hydrolysis of the terminal chitin chain, endochitinases cleave at internal sites (reviewed by Javed *et al.*, 2013).

The viral *chitA* encodes for a 58 kDa late protein containing both high endo- and exochitinase activity, which remain high throughout the rest of the viral replication cycle.

Possibly, baculoviruses acquired the *chitA* gene from bacteria via horizontal gene transfer (Hawtin *et al.*, 1995). High levels of ChitA activity were retained between pH 3.0-10.0 (Hawtin *et al.*, 1997).

Amino acids within ChitA crucial for liquefaction of *T. ni* larvae were assigned to the highly conserved aspartic acid 311 and glutamate (Glu, E) 315 residues, which most likely represent the active coding site of AcMNPV ChitA. Viruses containing a mutation in both ChitA D311G and E315G had a reduced chitinolytic activity and they failed to liquefy *T. ni* larvae. Mutation of the glutamate alone decreased exochitinase activity, delayed endochitinase activity and liquefaction of the larvae. Surprisingly, when only the aspartic acid residue was mutated exochitinase activity was reduced, whereas endochitinase activity was enhanced. Furthermore, mutagenesis of ChitA D311G had no effect on larvae liquefaction (Thomas *et al.*, 2000).

The viral *chitA* localises in the endoplasmatic reticulum (ER) of virus-infected cells for which it requires the KDEL signal peptide at the C-terminus (Thomas *et al.*, 1998; Saville *et al.*, 2002). Deletion of the ChitA KDEL motif resulted in the redistribution of ChitA to the cytoplasm in virus-infected cells. However, deletion did not compromise ChitA activity neither exo- nor endochitinase activity. On the contrary, KDEL motif deletion of ChitA resulted in a reduced lethal time and viral dose in virus-infected *T. ni* larvae. Moreover, larvae infected with a *chitA* KDEL deletion virus were liquefied about 24 h earlier than larvae infected with wild-type AcMNPV (Saville *et al.*, 2002, 2004).

In addition, P10 seems to be linked with viral ChitA as a delayed extracellular ChitA activity was observed when cells were infected with a *p10* deletion mutant *in vitro*. Furthermore, liquefaction of insect larvae was delayed by one day after infection with the same mutant virus (Thomas *et al.*, 1998).

a. Cathepsin

The viral Cath is a protease with the greatest enzymatic activity under acidic conditions (pH 5.0). Its protease activity was reduced in the presence of the cysteine protease inhibitors leupeptine and E64 but not serine protease inhibitors and was furthermore enhanced in the presence of Urea. These characteristics are typical for mammalian cathepsin-L-like proteases (Slack *et al.*, 1995). However, its substrate specificity more closely represents a cathepsin B protease (Brömme and Okamoto, 1995).

The gene *cath* is expressed as a precursor (prepro) enzyme (Fig. 1.8). The 22 amino acids of the prepro-Cath cause entry and localisation of the enzyme inside the ER of virus infected cells (Hodgson *et al.*, 2009, 2011). Inside the ER, the signal peptide is cleaved off resulting in a 32 kDa pro-enzyme, which is subsequently glycosylated at asparagine 65

resulting in a 35.5. kDa pro-Cath (Slack *et al.*, 1995; Hom *et al.*, 2002; Katsuma *et al.*, 2009). The viral ChitA may serve as a molecular chaperon for the proper folding of Cath in the ER. Viral pro-Cath was aggregated in the ER of virus-infected cells and did not undergo processing both *in vitro* and *in vivo*, when either *chitA* was replaced with a *lacZ* cassette or N-linked glycosylation was inhibited by tunicamycin (Hom and Volkman, 2000). On the contrary, when the *chitA* orf was deleted from the viral genome, Cath became processed and matured in a catalytically active enzyme (Hodgson *et al.*, 2013). Nevertheless, both fluorescence microscopy and pull down assays indicated that ChitA and Cath interact directly with each other in the ER of virus-infected cells (Hodgson *et al.*, 2011). The proenzyme pro-Cath remains inactive until cell death, when it is processed into the 27.5 kDa mature and catalytically active Cath, which is associated with dead cells *in vitro* (Hom *et al.*, 2002). Furthermore, it may be involved in actin and host cell cytoskeleton degradation (Lanier *et al.*, 1996).

Deletion of *cath* from the AcMNPV genome resulted in a delay of polyhedra release and cell lysis in infected TN368 cell (Hom *et al.*, 2002). A delayed release of OBs was also observed when *cath* was knocked out from the *B. mori* genome both *in vitro* and *in vivo* (Katsuma *et al.*, 2009).



Figure 1.8 Processing of cathepsin

The viral cathepsin (Cath) is expressed as a precursor (prepro-Cath) inactive protease, which is processed into a proenzyme (pro-Cath) inside the ER of virus-infected cells. Inside the ER the pro-Cath is glycosylated at the proenzyme region (glycopro-Cath) at asparagine⁶⁵ (N). After cell death, the proenzyme becomes catalytically active and matures into Cath, which is required for host cadaver liquefaction.

1.6.2 Persistent baculovirus infections

The main route of baculovirus transmission *in vivo* is horizontally via the highly robust and long-term resistant OBs resulting in liquefaction of their host (Fig. 1.3; Introduction 1.5). However, the host can survive the initial virus infection resulting in a sublethal infection

with reduced host fitness. Sublethal infections have the potential to spread vertically from parent to offspring either transovarially from the maternal parent to the embryo within the egg or transovum by the contamination of the exterior egg. Vertical transmission relies on replication and often takes place when hosts are rare and the population density is low (Fuxa and Richter, 1991; Fuxa *et al.*, 2002; Khurad *et al.*, 2004; Cabodevilla *et al.*, 2011).

Alternatively, vertical transmission can result in a persistent virus infection with no cost on host fertility and development (Burden *et al.*, 2003). In contrast to a latent virus infection, viral genes are expressed at low-levels and progeny virus is generated. Persistent virus infection can either result in a resistance against infection with a second virus (superinfection) or no immunity against superinfection (reviewed by Bonsall *et al.*, 2005).

Both homologous and heterologous interference was analysed *in vitro* by fluorescently labelled alphabaculoviruses. Superinfection was dramatically reduced when BVs began to egress from virus-infected cells both with homologous and heterologous baculoviruses. The exclusion of superinfection was likely due to actin rearrangements during virus infection as the inhibition of actin polymerisation by cytochalasin D enabled infections with a second virus (Beperet *et al.*, 2014).

An advantage of virus-persistence is that the virus can be carried great distances during host migrations and thereby exploiting new habitats. Occasionally, outbreaks of persistent viruses in natural insect populations occur, which might happen when the insect field population is high (Fuxa *et al.*, 1999; Cooper *et al.*, 2003).

The first molecular characterisation of a persistently-infected insect with baculovirus was performed in a laboratory strain of the cabbage moth *Mamestra brassicae* (Hughes *et al.*, 1993). A persistent MbNPV virus was found in the fat body in each stage of the insect life cycle- the egg, the larva, the pupa and the adult, continuously expressing viral proteins at low levels. Interestingly, MbNPV was activated leading to a fully overt lethal infection when *M. brassicae* larvae were challenged with either the closely related virus *Panolis flammea* NPV (PfNPV) or the distantly related AcMNPV (Hughes *et al.*, 1993, 1997).

A persistent *M. brassicae* infection was not only found under laboratory conditions but was also frequently present in natural *M. brassicae* populations in the United Kingdom (UK). Baculovirus DNA from either MbNPV or MbNPV and PfNPV was detected in all field-collected *M. brassicae* samples from ten different sites in the UK suggesting that persistent, asymptomatic infections are common in the field (Burden *et al.*, 2003). Sequencing of the highly conserved *polh* region and the more variable *ie-1* region of different persistently infected *M. brassicae* populations revealed only little genetic variation between the insect populations and thereby making it impossible to draw conclusions if

the persistently infected *M. brassicae* populations inside the UK were a result of a single or multiple infections. However, as persistent PfNPV was found in one population, the establishment of persistent infections must have occurred at least twice in *M. brassicae* inside the UK (Burden *et al.*, 2006).

In addition, persistent virus infections have been reported for other baculoviruses, such as *Choristoneura fumiferana* (CfMNPV; Kemp *et al.*, 2011), *S. exigua* (SeMNPV; Murillo *et al.*, 2011) or *S. exempta* (SpexNPV; Vilaplana *et al.*, 2010).

In a baculovirus *in vitro* model, it was hypothesised that deletion of the viral anti-apoptotic gene *p35* from the AcMNPV genome resulted in a persistent baculovirus infection in *Sf9* cells. The viral genes from the persistent virus were still expressed and BVs were still propagated indicating a fine balance between viral gene expression and the apoptotic pathway. Moreover, the persistently infected cells were resistant to superinfection with either wild-type AcMNPV or AcMNPV containing a *p35* deletion (Lee *et al.*, 1998).

In addition, it was reported that after a series of undiluted virus passages persistently infected SeMNPV cells were generated that only harboured a partial SeMNPV genome. They were resistant to infection with the homologous SeMNPV, whereas replication of the heterologous AcMNPV seemed to be unaffected (Weng *et al.*, 2009).

How persistent infections both *in vitro* and *in vivo* are maintained and regulated are still poorly understood and remain a subject of research. However, if the mechanism of the host and virus balance in a persistent state could be understood in more detail, new possibilities would arise in the context of the continuous production of recombinant proteins.

1.7 Baculovirus expression vectors

Baculoviruses have been widely used as recombinant protein expression vectors for over 35 years. A key experiment for the development of baculovirus expression vectors (BEV) was performed by Smith *et al.*, 1983a. They showed that site-specific mutations or the deletion of *polh* did not interfere with the production of progeny budded viruses *in vitro*. In the same year they published the first report of the use of baculoviruses as expression vectors by taking advantage of the knowledge that *polh* is not essential in cell culture. They cloned the coding sequence of the human beta Interferon (IFN- β) with its 3'untranslated region (UTR; harbouring the 3'PAS) into the *Bam*HI cloning site of the *Eco*RI-I fragment from AcMNPV, which contained the *polh* fragment. Recombinant viruses were produced by homologous recombination of the obtained transfer plasmid with the viral DNA, subsequent isolation by plaque purification and screening for polyhedrin-negative plaques (Fig. 1.9A).

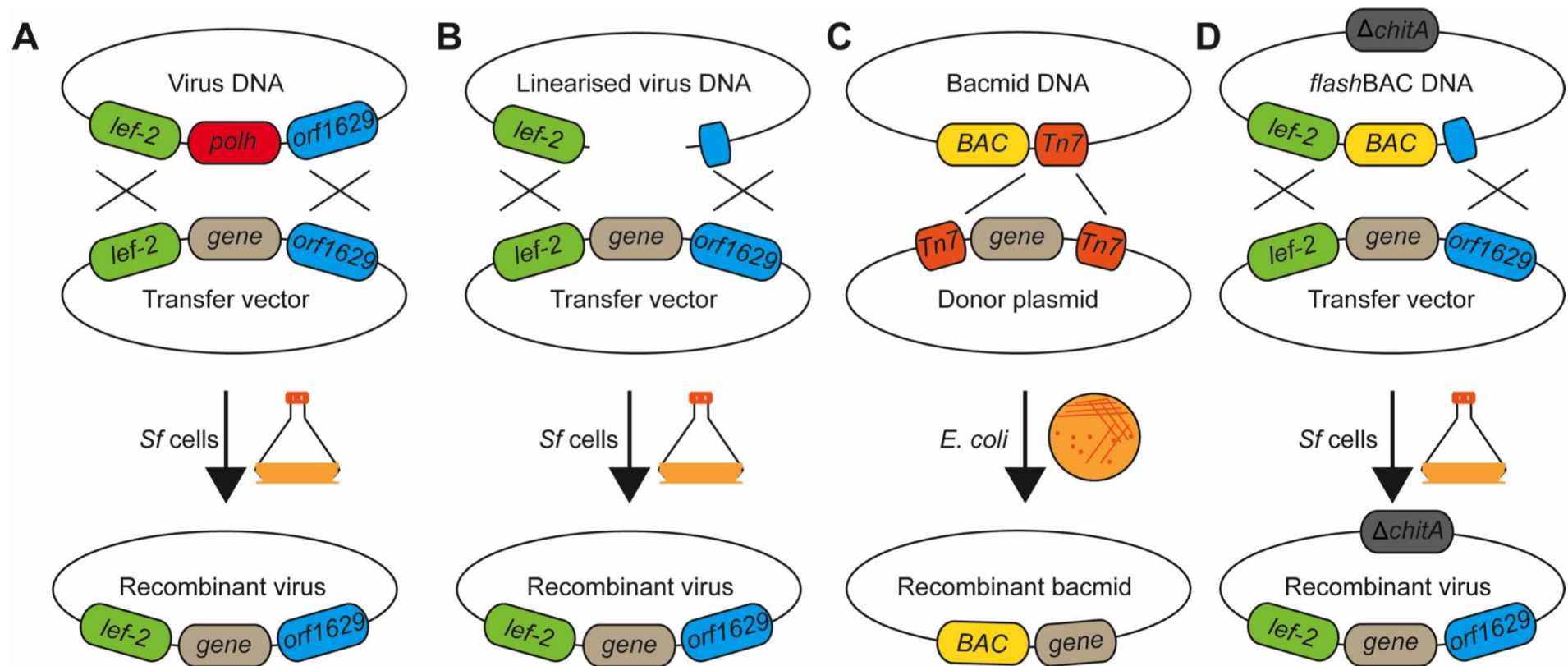


Figure 1.9 Generation of baculovirus expression vectors

A) Historically recombinant baculoviruses were generated by homologous recombination of viral DNA with a transfer vector encoding for the protein of interest under the *polh* promoter in insect cells and a subsequent purification of polyhedrin-negative plaques. B) Linearisation and restoration of the essential viral *orf1629* during homologous recombination dramatically increased recombinant virus production. C) The bacterial artificial chromosome gene (bacmid) technology relies on the site-directed transposition of a foreign gene into the *polh* locus in *E. coli* using a donor plasmid. D) The *flashBAC*TM DNA (OET Ltd.) not only lacks the full essential viral *orf1629* but also substitutes the *polh* gene with the *BAC* gene: Homologous recombination of *flashBAC*TM DNA with a transfer vector in insect cells fully recovers recombinant viruses without the need of any antibiotic selection. Additionally, the viral DNA can be modified [e.g. deletion of the viral *chitinase A* (*chitA*)] to further enhance recombinant protein production.

The recombinant virus generated, in which the sequence of the human interferon IFN- β was fused to the AcMNPV *polh* promoter produced normal levels of BVs demonstrating that the foreign protein did not interfere with viral replication. More importantly, this virus efficiently produced and secreted human IFN- β into the cell culture medium of infected insect cells. Furthermore, the secreted IFN- β was glycosylated and the signal peptide was cleaved demonstrating that mature IFN- β was produced (Smith *et al.*, 1983b). Subsequently, this technology was applied to drive foreign gene expression under the second viral very late promoter *p10* (Knebel *et al.*, 1985; Vlak *et al.*, 1990).

However, it was also noted that some recombinant proteins that require extensive posttranslational modifications are better produced in the late phase of infection. For example, the juvenile hormone esterase (JHE) or the human urokinase were expressed to higher yields under the viral basic promoter *p6.9* than the viral very late promoters *p10* or *polh* although the very late promoters are stronger (Bonning *et al.*, 1994; Lawrie *et al.*, 1995).

Unfortunately, the generation of recombinant virus by homologous recombination was very inefficient as only 0.5% of the plaques contained the desired recombinant virus (Smith *et al.*, 1983b; Pennock *et al.*, 1984). A major improvement of the BEV system was achieved when it was demonstrated that the linearisation of baculovirus DNA prior to co-transfection with the appropriated transfer vector greatly improved the yields (raised to ~30%) of recombinant viruses. The linearised viral DNA itself was 15-150-fold less infectious than the circular DNA and therefore highly reduced background contamination of the recombinant virus with the parental virus (Kitts *et al.*, 1990).

This approach was most effective (almost 100%) when the *polh* upstream gene *orf603* and downstream gene *orf1629* (*pp78/83*) inside the baculovirus expression vector were disrupted (Fig. 1.9B; Kitts and Possee, 1993). Whereas it is believed that ORF603 is nonessential for viral replication, the viral structural protein PP78/83 acts as an actin promoting factor required for viral transport to the nucleus after entry (Goley *et al.*, 2006). Even in the case if the linearised viral DNA religates, the virus cannot establish an infection and therefore this method avoids the need for several rounds of plaque purification (Kitts and Possee, 1993). Introduction of a *lacZ* gene inside the native *polh* region allowed a simple blue/white selection for baculovirus vectors containing the gene of interest. The linear baculovirus was commercialised and is available as BacPAK6 [Oxford Expression Technologies (OET) Ltd.] or Bac-N-Blue DNA (Invitrogen™).

Another milestone in the BEVS technology was accomplished by the construction of a bacterial artificial chromosome (bacmid), which can replicate in *Escherichia coli*. Bacmids

are fully infectious in insect cells and thereby act as shuttle vectors. In addition to the AcMNPV genome sequence, bacmids contain a mini-F-replicon allowing replication in *E. coli*, Kanamycin/Chloramphenicol as selection markers and an att7n site, which is a Tn7 transposition site. All those modifications were inserted into the *polh* region of AcMNPV. Foreign genes can be inserted into the att7n site inside the AcMNPV *polh* region by site-directed bacterial transposition using a donor plasmid that encodes for the gene of interest flanked by Tn7 sites (Fig. 1.9C; Luckow *et al.*, 1993). This technology is commercially available and widely known as the Bac-to-Bac® expression system (Invitrogen™).

On the same principle of using a transfer shuttle bacmid relies the Red/ET or λ -mediated homologous recombination in *E. coli*, which for example could be used to generate virus knock-out mutants by replacing the gene of interest with a selection marker (Fig. 1.10). In this system, the λ prophage genes are expressed by the temperature sensitive (ts) plasmid pRed/ET, which encodes for RecE, a 5'→3' exonuclease and RecT, a DNA annealing protein under the control of the L-arabinose inducible promoter pBAD. The Red/ET protein pair mediates DNA double-stranded break repair and recombination through homology regions (Zhang *et al.*, 1998; Muyrers *et al.*, 1999). The Red/ET mediated recombination has been commercialised by Gene Bridges GmbH.

Further improvements to the BacPAK6 system were made by the combination of the bacmid and the homologous recombination technology resulting in the commercial available *flashBAC*™ system (OET Ltd.), allowing high through put expression of recombinant proteins. The foreign gene targets the bacterial replicon inside the *polh* locus and selection is provided by repairing the viral *orf1629* genes as in the linear approach without the need for any antibiotic selection (Fig. 1.9D; Possee *et al.*, 2008).

For the optimisation of recombinant protein production, both the Bac-to-Bac® and *flashBAC*™ technology were modified by the deletion of some viral genes. Simultaneous deletion of both the viral *chitA* and *cath* in bacmids had positive effects on the integrity of both intracellular and secreted recombinant protein. Deletion of these viral genes protected the secreted recombinant protein from degradation and enabled higher production of the foreign protein (Kaba *et al.*, 2004). Additional deletion of the nonessential viral genes *p26*, *p74* and *p10* in the context of *chitA* and *cath* deletion increased expression of cytosolic recombinant proteins (Hitchman *et al.*, 2009).

The BEV system is nowadays widely accepted providing the capacity for the production of very high yields of proteins including plasma membrane localised proteins, secreted proteins, oligomeric proteins and proteins that require extensive posttranslational modifications (reviewed by Possee *et al.*, 2010; van Oers, 2011; van Oers *et al.*, 2014).

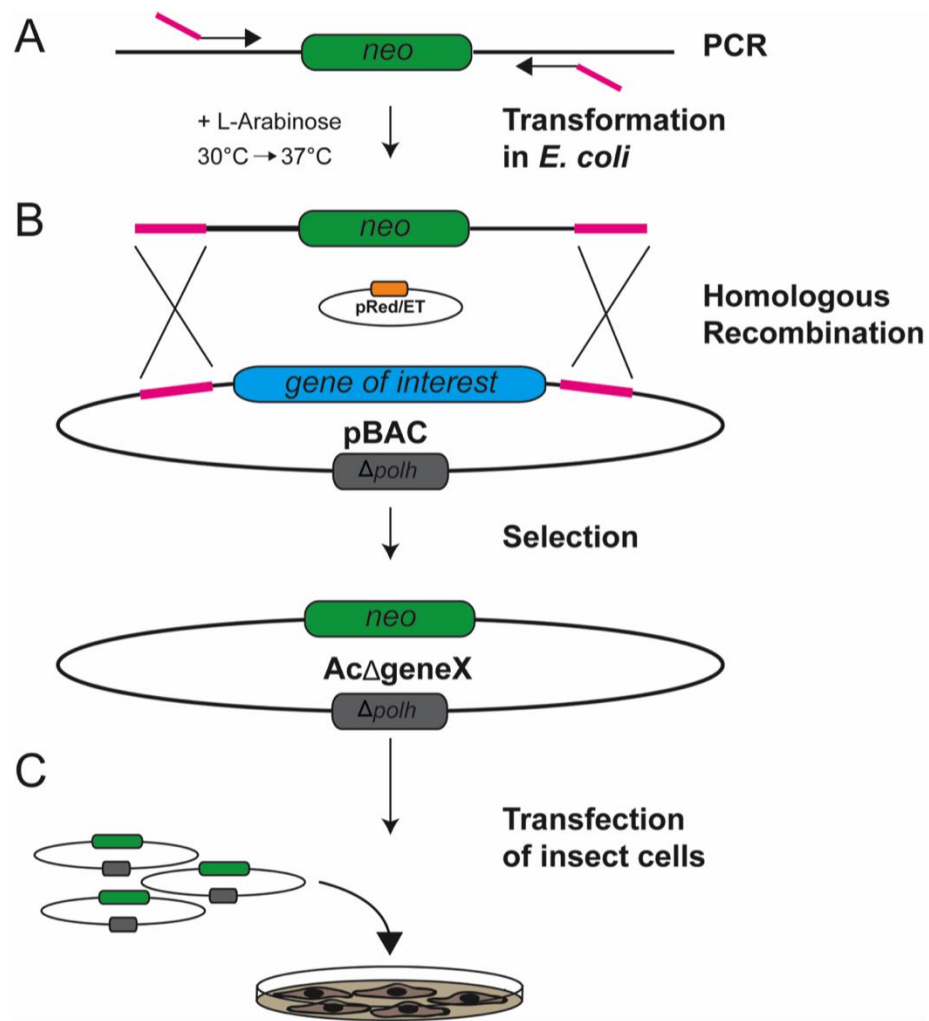


Figure 1.10 Generation of recombinant baculovirus by Red/ET homologous recombination

A) To generate knock-out bacmids (BAC) the *neomycine* (*neo*)/*kanamycine* (*kana*) cassette is amplified with primers containing the homology region of the gene of interest at their 5'ends. B) Expression from the recombination-mediated proteins from the temperature-sensitive pRed/ET in *E. coli* is induced by L-arabinose. After shifting the temperature to 37°C, the PCR product is electroporated into the *E. coli* cells, where homologous recombination occurs replacing the gene of interest with the selection marker *neo/kana*. C) Viruses can be generated by transfection of insect cells with the knock-out bacmid.

1.8 Applications of baculovirus expression vector systems

The baculovirus expression vector system (BEVS) is a well-defined system, which allows the insertion of large pieces of foreign DNA and has the capacity to produce very high yields of foreign protein. Baculoviruses specifically infect arthropods but they are able to transduce mammalian cells allowing recombinant gene expression from mammalian promoters. Hence, the BEVS has been successfully adapted to produce hundreds, possibly thousands of diverse recombinant proteins including multi-protein protein

complexes for medical utilisation such as safe vaccine production and gene therapy (Table 1.3; reviewed by Assenberg *et al.*, 2013; Contreras-Gómez *et al.*, 2014; van Oers *et al.*, 2014).

The first recombinant vaccine for humans produced by BEVS was approved in September 2007 in the European Union (EU) by the European Medicines Agency (EMA) and in October 2009 in the US by the FDA. It is a bivalent vaccine against the two most common high-risk HPV types 16 and 18 and was commercialised by GlaxoSmithKline plc as Cervarix® (Table. 1.3). The BEVS produce the HPV16 and HPV18 major surface proteins L1 in *T. ni* cells (Harper, 2008; Hashimoto *et al.*, 2012) that spontaneously self-assemble into highly immunogenic virus-like particles (VLPs), which do not contain the genome of the pathogen (Le Cann *et al.*, 1994; Harro *et al.*, 2001).

Table 1.3 Currently* approved recombinant vaccines for humans produced by BEVS

Name	Company	Licensed	Reference
Cervarix ®	GlaxoSmithKline plc	2007 (EMA) 2009 (FDA)	(Harper, 2008)
Provenge® (Sipuleucel-T)	Dendreon Pharmaceuticals Inc.	2013 (EMA) 2010 (FDA)	(Kantoff <i>et al.</i> , 2010)
Flublok®	Protein Sciences Corporation	2013 (FDA)	(Cox and Hollister, 2009)

*December 2017

Another example of recombinant vaccine produced by BEVS is the vaccine Flublok® against the seasonal influenza, which was licensed by the FDA in January 2013 (Cox and Hollister, 2009). The BEVS avoids the classical way of vaccine development in chicken eggs. Instead, the Influenza glycoprotein HA is produced under the baculovirus *polh* promoter by homologous recombination in *Sf9* cells (Cox and Hollister, 2009; reviewed by Cox and Hashimoto, 2011; Cox, 2012).

In addition to the recombinant vaccines for humans, a number of recombinant vaccines based on BEVS for veterinary utilisation have been approved by both the FDA and EMA, for example a vaccine against the classical swine fever (reviewed by van Oers *et al.*, 2014).

Importantly, the vaccine market is expected to increase dramatically and it is estimated that the market will reach ~ \$50 billion by 2022 with a compound annual growth rate of 7.5% (Vaccine Market Report). In addition, the market for VLP-based vaccines is the most appealing and most cost efficient approach and it is further considered as safe. Major markets for VLP-based vaccines include pharmaceutical companies such as Merck & Company, Inc., GlaxoSmithKline plc or MedImmune LLC. (VLP Market Report). Currently ~60 VLP based vaccine are in clinical trials but even more candidates have been

published recently, e.g. against Hepatitis C virus (Wei *et al.*, 2018), Coxsackievirus (Zhou *et al.*, 2018), Nipah virus (Walpita *et al.*, 2017), Porcine circovirus type 2 (Chen *et al.*, 2018) or Foot and Mouth Disease virus (Veerapen *et al.*, 2018). However, the vaccine market is still high in demand for new vaccine types and more divergence in demand for mature and combination vaccine types (WHO Vaccine Report). Therefore, it is likely that even more VLP-based vaccines will gain approval by both the FDA and EME.

Hence, improvements of the BEVS might be directly applied in the commercial development of vaccine production. This will allow a) a more time efficient vaccine production against a number of diseases, resulting in b) a faster reaction after a pandemic outbreak and will c) reduce manufacturing costs. A more cost absence of economic incentives efficient production will lead to d) an increase in candidate vaccine development.

1.9 Research aims

Baculoviruses have been used for recombinant protein expression for decades and have been successfully applied for vaccine production. However, very little is known about how the late and very late phases of baculovirus infection are regulated (Fig. 1.2). What causes the transition from the viral late to the very late phase of infection? A better understanding of what virus genes are expressed throughout this cycle may enable the manipulation of viral gene expression, which could have significant benefits for the expression of certain types of recombinant proteins, including vaccines.

However, *in vivo*, baculoviruses are often maintained as persistent virus infections. Insects infected with a persistent baculovirus do not liquefy and maintain a specific balance of virus and host gene expression (Burden *et al.*, 2003, 2006). *In vitro*, insect cell lines harbouring a persistent baculovirus do not lyse (Hughes *et al.*, 1993, 1997). This raised the question of how gene expression is regulated in a persistent baculovirus infection and how this differs from a lytic baculovirus infection.

Thus, the main aim of the study is to understand how late and very late gene expression in both lytic and persistent baculovirus infections is regulated. In order to do this, the study is divided into the following parts:

The first three results chapters aim to understand how AcMNPV gene expression is regulated in a lytic infection, whereby each chapter aims to describes the involvement of different viral proteins: Cath, ChitA, LEF-2 and VP39.

It was noticed that P10 might get processed at the very end of the viral replication cycle (Weyer *et al.*, 1990) by Cath (R. D. Possee, personal communication). As it was assumed

previously that Cath might be important for cell lysis to occur (Hom *et al.*, 2002) and it was shown that P10 is required for polyhedra release (Williams *et al.*, 1989), it was hypothesised that processing of P10 might be required for virus-induced nuclear disintegration. Moreover, as Cath and ChitA often act together and have similar functions, e.g. during liquefaction of the larvae (Slack *et al.*, 1995; Hawtin *et al.*, 1997) Chapter 3 aims

- To examine the potential interaction between P10 with the viral enzymes cathepsin and chitinase A and to determine the role of cathepsin in virus-induced cell lysis.

One protein that was already implicated to play a role in *p10* and *polh* expression by mutagenesis studies is LEF-2 (Merrington *et al.*, 1996). Originally, LEF-2 was identified to be essential for virus replication by acting as a primase associated protein (Passarelli and Miller, 1993; Mikhailov and Rohrmann, 2002). Hence, Chapter 4 aims

- To determine the apparent dual function of LEF-2 in AcMNPV DNA replication and viral late/very late gene expression

Although VP39 has been mainly recognised as the major structural protein, BrdU mutagenesis studies in BmNPV implicated that it may also play a role in viral very late gene expression (Katsuma and Kokusho, 2017). Furthermore, the knock-down of *vp39* by RNAi in AcMNPV-infected cells resulted in a reduced accumulation of P10 on a protein gel (G. W. Blissard, personal communication). Therefore, the aim of Chapter 5 is:

- To investigate the effects of a *vp39* knock-down on viral gene expression

Moreover, our laboratory was able to establish a persistently infected insect cell line, the C20 cell line (R. D. Possee, personal communication). This cell line has been maintained for several years and permits analysis of baculovirus persistence in a predefined *in vitro* system. A collaboration with Gary W. Blissard's lab at Boyce Thompson Institute (BTI) in the US resulted in the opportunity to characterise the transcriptome of the C20 cell line by strand specific RNA-sequencing (RNA-seq). Hence, the following two results chapters aim to understand how gene expression is regulated in a persistently-infected insect cell line and to compare these results with an overt infection, more specifically:

- To analyse the phases of viral gene transcription in a productive AcMNPV-infection *in vitro* and to compare the results with the persistently-infected C20 cell line (Chapter 6).
- To determine the consequences of the C20 cell line evolution on host gene expression (Chapter 7).

Unfortunately, recent investigations to recreate the C20 cell line or other insect cell lines harbouring a persistent baculovirus failed. Therefore, the aim of the last results chapter is

- To reestablish a newly persistently infected insect cell line and to initially characterise this cell line (Chapter 8).

Overall, this thesis aims to describe multiple approaches to the study of baculovirus gene expression.

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Chapter Two:

Materials and Methods

2.1 Materials

2.1.1 Insect cell lines and cell culture reagents

The experiments described in this study utilised the following insect cell lines: *Sf9* cells, which are a clonal cell line derived from IPLB-Sf21 (*Sf21*) cells. *Sf21* cells were isolated from the fall army worm (Vaughn *et al.*, 1977). *T. ni* High Five™ (BTI-TN-5B1-4, *T. ni* Hi5™) cells derived from the cabbage looper (Granados *et al.*, 1994) and its subclonal cell line Tnms42 (Chen *et al.*, 2013). For details on the newly established AcLEF-2^{C84A}/Hi5 cell line see Chapter 8. All cell lines were maintained as described in Table 2.1 and incubated at 28°C.

Table 2.1 Insect cell lines

Cell line	Type	Medium	Seeding density	Max. Passage density	Source
AcLEF-2 ^{C84A} /Hi5	Adherent	ESF 921 (Expression Systems™)	1:3-1:10	At confluence	This thesis
<i>Sf9</i>	Suspension	ESF 921	1-3x 10 ⁵ cells/ml in shake culture (135 rpm)	3-8x 10 ⁶ cells/ml	Invitrogen™
<i>Sf21</i>	Suspension	TC 100 (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma-Aldrich)	4-5x 10 ⁵ cells/ml in spinner culture (80-90 rpm)	1-2x 10 ⁶ cells/ml	R. D. Possee (OET Ltd., Oxford, UK)
<i>T. ni</i> Hi5™	Suspension	ESF 921	2-5x 10 ⁵ cells/ml in shake culture (135 rpm)	3-8x 10 ⁶ cells/ml	Invitrogen™
Tnms42	Adherent	Sf900III (Gibco®)	1:3-1:10	At confluence	G. W. Blissard (Boyce Thompson Institute-BTI, Ithaca, NY, US)

2.1.2 Viruses

All viruses generated or utilised in this study are presented in Table 2.2. Viruses were supplemented with 2.5% volume/volume (v/v) fetal calf serum (FCS) and working stocks were stored at 4°C, whereas for long-term storage the inocula were kept at -80°C.

Table 2.2 Viruses

Virus	Description	Source	Reference	Chapter
AcMNPV C6	Plaque isolate of the wt prototype type I alphabaculoviruses	R. D. Possee (OET Ltd.)	(Possee, 1986; Possee <i>et al.</i> , 1991)	3,4,6
AcΔcath	Recombinant AcMNPV lacking the <i>cath</i> and <i>polh</i> coding regions	This thesis	-	3
AcΔcath ^{polh+}	Recombinant AcMNPV lacking the <i>cath</i> orf, <i>polh</i> was reinserted into its natural locus	This thesis	-	3

Table 2.2 Continued: Viruses

AcΔcathΔp10	<i>flashBAC ULTRA™</i> containing deletions of <i>p10</i> , <i>p74</i> , <i>p26</i> , <i>chitA</i> , <i>cath</i> ; coding region of <i>polh</i> into its native locus	R. D. Possee (OET Ltd.)	(Hitchman <i>et al.</i> , 2009)	3
AcΔchitA	Recombinant AcMNPV lacking the <i>cath</i> orf, <i>polh</i> was reinserted into its natural locus	R. D. Possee (OET Ltd.)	-	3
AcΔlef-2	AcMNPV bacmid, in which the coding sequence of <i>lef-2</i> was exchanged by a <i>neo</i> expressing cassette	A. Chambers (OET Ltd.)	-	4
AcΔlef-2 ^{rescue}	AcMNPV bacmid, in which the coding sequence of <i>lef-2</i> was removed and afterwards rescued with AcMNPV C6 <i>lef-2</i> including the <i>orf603</i> , <i>polh</i> encoding fragment	This thesis	-	4
AcΔp10. <i>lacZ</i>	Recombinant AcMNPV lacking the <i>p10</i> orf; <i>lacZ</i> was inserted into the <i>p10</i> locus under the <i>p10</i> promoter	L. P. Graves	(Graves, 2017)	3
AcΔpolh	<i>flashBAC PRIME™</i> containing a deletion of the <i>polh</i> coding region	R. D. Possee (OET Ltd.)	(Hitchman <i>et al.</i> , 2009)	4
AcGFP	<i>flashBAC ULTRA™</i> containing deletions of <i>p10</i> , <i>p74</i> , <i>p26</i> , <i>chitA</i> , <i>cath</i> ; coding region of <i>gfp</i> was inserted under <i>polh</i> promoter	R. D. Possee (OET Ltd.)	(Hitchman <i>et al.</i> , 2009)	4
AcLEF-2 ^{C84A}	Recombinant AcMNPV generated by AcΔlef-2 rescue; encoded for LEF-2 ^{C84A} ; <i>orf603</i> and <i>polh</i> were reinserted into their native loci	This thesis	-	4, 8
AcLEF-2 ^{C143A}	Recombinant AcMNPV generated by AcΔlef-2 rescue; encoded for LEF-2 ^{C143A} ; <i>orf603</i> and <i>polh</i> were reinserted into their native loci	This thesis	-	4
AcLEF-2 ^{C146A}	Recombinant AcMNPV generated by AcΔlef-2 rescue; encoded for LEF-2 ^{C146A} ; <i>orf603</i> and <i>polh</i> were reinserted into their native loci	This thesis	-	4
AcLEF-2 ^{C150A}	Recombinant AcMNPV generated by AcΔlef-2 rescue; encoded for LEF-2 ^{C150A} ; <i>orf603</i> and <i>polh</i> were reinserted into their native loci	This thesis	-	4
AcLEF-2 ^{C165A}	Recombinant AcMNPV generated by AcΔlef-2 rescue; encoded for LEF-2 ^{C165A} ; <i>orf603</i> and <i>polh</i> were reinserted into their native loci	This thesis	-	4
AcLEF-2 ^{C84S}	Recombinant AcMNPV generated by AcΔlef-2 rescue; encoded for LEF-2 ^{C84S} ; contained <i>orf603</i> and <i>polh</i> deletion	R. D. Possee (OET Ltd.)	(Allen, 2006)	4, 8
AcLEF-2 ^{C88S}	Recombinant AcMNPV generated by AcΔlef-2 rescue encoded for LEF-2 ^{C88S} ; contained <i>orf603</i> and <i>polh</i> deletion	R. D. Possee (OET Ltd.)	(Allen, 2006)	4
AcLEF-2 ^{C146S}	Recombinant AcMNPV generated by AcΔlef-2 rescue encoded for LEF-2 ^{C146A} ; contained <i>orf603</i> and <i>polh</i> deletion	R. D. Possee (OET Ltd.)	(Allen, 2006)	4
AcLEF-2 ^{A154T}	Recombinant AcMNPV; Amino acid 3548 in <i>lef-2</i> orf was changed to same mutation as in VLD1 (encoding for A154T)	This thesis	-	4
AcLEF-2 ^{D178N, polh-} Published as Aclef-2 ³⁶²⁰	Recombinant AcMNPV; Amino acid 3620 in <i>lef-2</i> orf was changed to same mutation as in VLD1 (encoding for D178N), contained <i>polh</i> deletion	R. D. Possee (OET Ltd.)	(Merrington <i>et al.</i> , 1996)	4
AcLEF-2 ^{D178N}	Recombinant AcMNPV, <i>polh</i> was reinserted into its native locus in AcLEF-2 ^{D178N, polh-}	This thesis	-	4

Table 2.2 Continued: Viruses

AcHis. <i>p10</i>	Recombinant AcMNPV encoded for N-terminally 6x His-tagged P10	L. P. Graves	(Graves, 2017)	3
AcRP23. <i>lacZ</i>	Recombinant AcMNPV lacking the <i>polh</i> orf, <i>lacZ</i> was inserted into <i>polh</i> locus under <i>polh</i> promoter	R. D. Possee (OET Ltd.)	(Possee and Howard, 1987)	3, 4
AcUW1. <i>lacZ</i>	Recombinant AcMNPV lacking the <i>p10</i> orf, <i>lacZ</i> was inserted into <i>BglII</i> of <i>p10</i> locus under <i>p10</i> promoter, while the first 152 nt of <i>p10</i> were deleted, still contained the <i>p10</i> 5' leader sequence, 132 remaining nt at the 3' end and the 3' UTR; <i>polh</i> was retained at its native locus	R. D. Possee (OET Ltd.)	(Weyer <i>et al.</i> , 1990)	4
AcLEF-2 ^{A154T,D178N}	Recombinant AcMNPV expressing VLD1 <i>lef-2</i> , generated by rescuing AcΔ <i>lef-2</i> ; <i>polh</i> and <i>orf603</i> region was inserted into its native locus	This thesis	-	4
AcLEF-2 ^{A154T,D178N,polh-}	Recombinant AcMNPV expressing VLD1 <i>lef-2</i> , generated by rescuing AcΔ <i>lef-2</i> without reinserting <i>polh</i>	This thesis	-	4
Ac.mycLEF-2	Recombinant AcMNPV encoding for N-terminally c-MYC-tagged LEF-2	This thesis	-	4
VLD1	Recombinant AcMNPV generated by random mutagenesis of <i>Sf21</i> cells infected with AcUW1. <i>lacZ</i> ; Sequencing revealed that this virus encoded for the following amino acid changes at the <i>EcoR-I</i> region: Bro ^{L144F} , LEF-2 ^{A154T} , LEF-2 ^{D178N} , PK-1 ^{A6V} , PK-1 ^{T184H} , PK-1 ^{T185N} , PK-1 ^{M186Y} , PK-1 ^{H187A} , PK-1 ^{Y188R}	R. D. Possee (OET Ltd.)	(Merrington <i>et al.</i> , 1996)	4
VLD1 ^{EcoR-I}	Recombinant AcMNPV, in which the wt <i>EcoR-I</i> fragment was reinserted into VLD1; generated by co-transfection	This thesis	-	4
VLD1 ^{AcΔpolh}	Recombinant VLD1, which was rescued by co-transfection with AcΔ <i>polh</i>	This thesis	-	4
VLD1 ^{LEF-2}	Recombinant AcMNPV, in which wt <i>lef-2</i> was reinserted into VLD1, generated by co-transfection	This thesis	-	4

2.1.3 Plasmids

All plasmids that were used are referred to in Table 2.3. They were stored at -20°C.

Table 2.3 Plasmids

Plasmid	Description	Source	Reference	Chapter
pAcEI-I	Transfer vector encodes for the 7.3 kbp AcMNPV <i>EcoR-I</i> genome region; AcMNPV genome coordinates: 428-7,759	R. D. Possee (OET Ltd.)	(Possee and Howard, 1987)	4
pBS.SK+	Recombinant plasmid contains multiple cloning side, which is located within <i>lacZ</i> coding region allowing for blue-white screening of successfully insertion of gene(s) of interest	R. D. Possee (OET Ltd.)	-	4
pBS.SK+CB1	Transfer vector encodes for AcMNPV LEF-2, ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB1 ^{LEF-2,C84A}	Transfer vector encodes for AcMNPV LEF-2 ^{C84A} , ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB1 ^{LEF-2,C143A}	Transfer vector encodes for AcMNPV LEF-2 ^{C143A} , ORF603 and polyhedrin	This thesis	-	4

Table 2.3 Continued: Plasmids

pBS.SK+CB1 ^{LEF-2,C146A}	Transfer vector encodes for AcMNPV LEF-2 ^{C146A} , ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB1 ^{LEF-2,C150A}	Transfer vector encodes for AcMNPV LEF-2 ^{C150A} , ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB1 ^{LEF-2,C165A}	Transfer vector encodes for AcMNPV LEF-2 ^{A154T,D178N} , ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB2	Transfer vector encodes for AcMNPV LEF-2 ^{A154T,D178N} , ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB3	Transfer vector encodes for AcMNPV LEF-2 ^{C165A} , ORF603 and polyhedrin	This thesis	-	4
pBS.SK+CB4	Transfer vector encodes for AcMNPV LEF-2 ^{A154T}	This thesis	-	4
pBS.SK+CB5	Transfer vector encodes for upstream homologous region of AcMNPV <i>lef-2</i>	This thesis	-	4
pBS.SK+CB6	Transfer vector expresses 5'-myc-tagged <i>lef-2</i> , <i>orf603</i> and <i>polh</i>	This thesis	-	4

2.1.4 Competent bacterial strains

Bacterial cell lines utilised for the generation or amplification of plasmids are listed in Table 2.4. Bacterial stocks were stored in aliquots at -80°C to avoid multiple freeze and thaw cycles.

Table 2.4 Competent bacterial cells for transformation

Name	Genotype	Source
DH5α	F- ϕ 80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1	Invitrogen™
DH10B	F ⁺ mcrA Δ(mrr-hsdRMS-mcrBC) Φ 80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ ⁺ rpsL nupG	Invitrogen™

2.1.5 Antibiotics

Antibiotics for maintenance of bacteria are presented in Table 2.5. Both stock and working solutions were stored at -20°C.

Table 2.5 Antibiotics for bacteria maintenance

Antibiotic	Stock concentration	Working concentration
Ampicillin	100 mg/ml in distilled (d)H ₂ O	100 µg/ml
Chloramphenicol	20 mg/ml in absolute EtOH	15 µg/ml
Kanamycin	30 mg/ml in dH ₂ O	15 µg/ml
Tetracycline	10 mg/ml in absolute EtOH	3 µg/ml

2.1.6 Primers and probes

The DNA oligos used in this study are listed in Table 2.6. Oligo primers were obtained from Eurofins MWG Operon (Germany) and dissolved in dH₂O. Dual labelled probes were purchased from either Eurofins MWG Operon or Eurogentec (Belgium) and dissolved in 1x TE (Tris-Ethylenediaminetetraacetic acid; EDTA) buffer (pH 8, Table 2.10). All DNA oligos were diluted to stock concentrations of 100 pmol/µl and stored at -20°C.

Table 2.6 Custom DNA oligos

DNA oligo name	Description	Sequence (5' → 3')	Chapter
Act-1	Forward primer for <i>Sf21</i> actin PCR (Allen, 2006)	GGACAGAAGGACTCGTACGTAGG	4
Act-2	Forward primer for <i>Sf21</i> actin PCR (Allen, 2006)	CAGCCTGGATGGCGACGTACAT	4
CB1	Forward primer for generating pBS.SK+CB1 ^{LEF-2,C84A} , amino acid change is underlined	AGAAACATT <u>GCT</u> ATGAAAGAATGCGTAGAAGG	4
CB2	Reverse primer for generating pBS.SK+CB1 ^{LEF-2,C84A}	CTTGCTGCGTTTTTTTAAATTGG	4
CB3	Forward primer for generating pBS.SK+CB1 ^{LEF-2,C143A} , amino acid change is underlined	TGGTTTCGGCTGCCAAGTGTGAAAAC	4
CB4	Reverse primer for generating pBS.SK+CB1 ^{LEF-2,C143A}	CGTTTGCAATGTAACAGTTTAG	4
CB5	Forward primer for generating pBS.SK+CB1 ^{LEF-2,C146A} , amino acid change is underlined	GAAAACCGAG <u>CT</u> TTAATCAAGGCTCTGACGCA	4
CB6	Reverse primer for generating pBS.SK+CB1 ^{LEF-2,C146A}	ACCACGTTTGCAATGTAACA	4
CB7	Forward primer for generating pBS.SK+CB1 ^{LEF-2,C150A} , amino acid change is underlined	GAAAACCGAG <u>CT</u> TTAATCAAGGCTCTGACGCA	4
CB8	Reverse primer for generating pBS.SK+CB1 ^{LEF-2,C150A}	ACACTTGGCACACGAAACCA	4
CB9	Forward primer for generating pBS.SK+CB1 ^{LEF-2,C165A} , amino acid change is underlined	CACGACTCCAAG <u>GCT</u> GTGGGTGAAGTC	4
CB10	Reverse primer for generating pBS.SK+CB1 ^{LEF-2,C165A}	GTTGTAGAAATGCGTCAGAGC	4
CB12	Forward primer binding inside 5' homologous region of <i>lef-2</i> , <i>Bgl</i> II digestion site is underlined, for generating pBS.SK+CB2 and pBS.SK+CB3	GGGAGATCTCCAAAAGAACTGCCGACTAC	4
CB13	Reverse primer binding inside 3' homologous region of <i>lef-2</i> , <i>Bgl</i> II digestion site is underlined, for generating pBS.SK+CB2	GGGAGATCTCAAATGTTCTTGTTGCGTTTG	4
CB14	Reverse primer binding inside 3' homologous region of <i>polh</i> , <i>Bgl</i> II digestion site is underlined, for generating pBS.SK+CB3	GGGAGATCTCTAATCGAAACCTATTTTACAATC	4
CB21	Forward primer, introduces <i>c-myc</i> -tag (small letters) at 3' <i>lef-2</i> , <i>Bgl</i> II digestion site is underlined	GGGAGATCTatggagcaaaagctcatttctgaagagga cttgAATTCTAGAATGGCGAATGCATCGTATA ACGTG	4
CB22	Reverse primer binds inside 3' homologous region of <i>polh</i> , <i>Not</i> I digestion site is underlined, for generating pBS.SK+CB6	GGGGCGGCCGCGGACGACGACGATAATC GGCC	4
CB23	Forward primer binds inside 3' <i>lef-2</i> homologous region, <i>Xho</i> I digestion site is underlined, for generating pBS.SK+CB5	GGGCTCGAGCGTAGTGACAACGATCACGC C	4
CB24	Reverse primer binds nt -27 until -1 from <i>lef-2</i> ATG, <i>Bam</i> HI digestion site is underlined, for generating pBS.SK+CB5	GGGGGATCCTACTTCGCGGCTTCTCGC	4
CB25	Forward primer, binds upstream of <i>ptp</i> , used for sequencing of <i>Eco</i> R-I region of VLD1	AGCGAGTTGAAGGATCATATTTAGTTGCG	4
CB26	Reverse primer, binds downstream of <i>orf603</i> , used for sequencing of <i>Eco</i> R-I region of VLD1	GAAAACAGTAAATACTTATTTATTTGC	4

Table 2.6 Continued: Customs DNA oligos

CB27	Forward primer, binds in nucleotide sequence of <i>orf1626</i> , upstream of <i>pk-1</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	CTGCACAACGGAAGGTCGTC	4
CB28	Reverse primer, binds downstream of <i>pk-1</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	CACGTAGAATTCTACTCGTAAACAAGTGG	4
CB31	Reverse primer, binds downstream of <i>ptp</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	GGTTCGCTGGAAGAAGCGCAAC	4
CB32	Forward primer, binds upstream of <i>Ac-bro</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	AGCCAGAGGTCACAAAATTGAAAGAC	4
CB33	Reverse primer, binds downstream of <i>Ac-bro</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	GTTGTAGCGGCGCATGTTCC	4
CB34	Forward primer, binds upstream of <i>ctx</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	CGCGAGCCATTTTGATACACGTGT	4
CB35	Reverse primer, binds downstream of <i>orf-4</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	CTCTAATGAGCGGACTCCACACG	4
CB36	Forward primer, binds upstream of <i>orf1626</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	CCTCAATAGATTGTGAAAATAGGTTTCG	4
<i>gp64</i> F	qPCR forward primer, binds within <i>gp64</i> ; used for virus titration by BaculoQUANT™ (Hitchman <i>et al.</i> , 2007; OET Ltd.)	CGGCGTGAGTATGATTCTCAA	3, 4, 8
<i>gp64</i> R	qPCR forward primer, binds within <i>gp64</i> ; used for virus titration by BaculoQUANT™ (Hitchman <i>et al.</i> , 2007; OET Ltd.)	ATGAGCAGACACGCAGCTTTT	3, 4, 8
<i>lef-2</i> ^{A154T} F	Forward primer to introduce A154T mutation in LEF-2, nucleotide sequence encoding for amino acid change is underlined	CCGATGTTTAATCAAGGATCTGACGCATTTCTAC	4
<i>lef-2</i> ^{A154T} R	Reverse primer to introduce A154T mutation in LEF-2, nucleotide sequence encoding for amino acid change is underlined	GTAGAAATGCGTCAGATCCTTGATTAAACATCGG	4
qPCR <i>actin</i> 1	qPCR forward primer, binds <i>Sf9</i> endogenous actin	GGACGACATGGAGAAGATCTG	4
qPCR <i>actin</i> 2	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	ACGATACCGGTGGTACGAC	4
qPCR <i>actin</i> 3	qPCR forward primer, binds <i>Sf9</i> endogenous actin	ATGAAGATCCTCACCGAG	4
qPCR <i>actin</i> 4	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	GGAAGTTCGTAGGACTTCTC	4
qPCR <i>actin</i> 5	qPCR forward primer, binds <i>Sf9</i> endogenous actin	ATCCGTAAGGACCTGTACGC	4
qPCR <i>actin</i> 6	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	CACATCTGCTGGAAGGTGGA	4
qPCR <i>actin</i> 7	qPCR forward primer, binds <i>Sf9</i> endogenous actin	GAT CTG GCA CCA CAC CTT CTA C	4
qPCR <i>actin</i> 8	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	GAGAGAAGATGACACAGATCATGTTCG	4
qPCR <i>actin</i> 9	qPCR forward primer, binds <i>Sf9</i> endogenous actin	CATCGAGCACGGTATCATC	4
qPCR <i>actin</i> 10	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	AGTCAGGAGGACTGGGTG	4

Table 2.6 Continued: Customs DNA oligos

qPCR <i>actin</i> 11	qPCR forward primer, binds <i>Sf9</i> endogenous actin	CAGGGTGTGATGGTTGGTAT	4
qPCR <i>actin</i> 12	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	ACATGATCTGGGTCATCTC	4
qPCR <i>actin</i> 13 ⁺	qPCR forward primer, binds <i>Sf9</i> endogenous actin	GGCCCAGAGCAAGAGAG	4
qPCR <i>actin</i> 14 ⁺	qPCR reverse primer, binds <i>Sf9</i> endogenous actin	CAGAGAGAGCACAGCCTG	4
qPCR <i>gp64</i> F	qPCR forward primer, binds within <i>gp64</i> ; used for virus titration by BaculoQUANT™	CGGCGTGAGTATGATTCTCAAA	4
qPCR <i>gp64</i> F2 ⁺	qPCR forward primer, binds within <i>gp64</i>	CTCGGTGCTCGACTTTGC	4
qPCR <i>gp64</i> F3	qPCR forward primer, binds within <i>gp64</i>	GTCAATCAAACAGTGTTGCGG	4
qPCR <i>gp64</i> F4	qPCR forward primer, binds within <i>gp64</i>	CGTGTCACCGACTCGG	4
qPCR <i>gp64</i> R	qPCR forward primer, binds within <i>gp64</i> ; used for virus titration by BaculoQUANT™	ATGAGCAGACACGCAGCTTTT	4
qPCR <i>gp64</i> R2	qPCR reverse primer, binds within <i>gp64</i>	GGACACTGTGCTTCATCG A	4
qPCR <i>gp64</i> R3	qPCR reverse primer, binds within <i>gp64</i>	TGG ACG CTG AGG GCA AC	4
qPCR <i>gp64</i> R4 ⁺	qPCR reverse primer, binds within <i>gp64</i>	GACGACACGGACGAGTG	4
qPCR probe <i>actin</i>	qPCR multiplex probe, binds actin sequence between QPCR <i>actin</i> 13 and 14	5'TAM- TGGCACCACACCTTCTACAACGAGCTGC- 3'BHQ2	4
qPCR probe <i>gp64</i>	qPCR multiplex probe, binds actin sequence between qPCR <i>gp64</i> F2 and R4	5'FAM- TTTGCGCGTGGTGAACGTAGACTTTT- 3'BHQ1	4
qPCR probe GP64 ^{titration}	qPCR probe, binds within <i>gp64</i> ; used for virus titration by BaculoQUANT™	5'FAM- TTTGCGCGTGGTGAACGTAGACTTTT- 3'TAM	3, 4, 8
RDP112*	Forward primer, binds downstream of <i>polh</i> ; to analyse successful insertion of <i>polh</i> region into recombinant virus	TGAGACGCACAACTAATATCACAAAC	3, 4
RDP113*	Reverse primer, binds upstream of <i>polh</i> ; to analyse successful insertion of <i>polh</i> region into recombinant virus	ATACGTACAACAATTGTCTGTAAATCAAC	3, 4
RDP541*	Forward primer, binds upstream of <i>polh</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	ACACCATTTTTTAGCAGCACCG	4
RDP544*	Reverse primer, binds downstream of <i>polh</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	AAGCTAGATTGCTGCTACAAG	4
RDP560*	Forward primer, binds upstream of <i>orf603</i> , used for sequencing of <i>EcoR</i> -I region of VLD1	GGCCGGCCTGAATAATAAAACAATTATAAA TGCTAAATT	4
RDP606*	Forward primer, binds downstream of <i>lef-2</i>	CCAAAAGAACTGCCGACTAC	4
RDP607*	Reverse primer, binds upstream of <i>lef-2</i>	CAAATGTTCTTGTGCGTTTGG	4
RDP712*	Reverse primer, binds inside <i>orf1626</i> orf, used for sequencing of <i>EcoR</i> -I region of VLD1	AAACGATAGCGAAATTACAATGC	4
RDP745*	Reverse primer, binds upstream of <i>cath</i>	TCCAGAGCGCTACGCTCTACC	3

Table 2.6 Continued: Customs DNA oligos

RDP944*	Reverse <i>neo</i> primer, <i>neo</i> specific sequence is underlined, contains downstream homologous region of <i>cath</i> , used for AcΔ <i>cath</i> generation	TGAGCTACTGAGACGATATGATTATGTTCC AAATAGCGAGTGTGTTGAGATCAGAAGAAC TCGTCAAGAAGGCG	3
RDP945*	Reverse <i>neo</i> primer, <i>neo</i> specific sequence is underlined, contains upstream homologous region of <i>cath</i> , used for AcΔ <i>cath</i> generation	ATGAACAAAATTTTGTGTTTATTTGTTTGTGT ACGGCGTTGTAAACAGCGCTGGACAGCAA GCGAACCGGAATTGC	3
RDP1106*	Forward primer for <i>fp25k</i> amplification for dsRNA synthesis, T7 promoter specific sequence is underlined; utilised for <i>fp25k</i> sequencing	TTAATACGACTCACTATAGGGACGTCTGGC AAGAATCACAAC	8
RDP1107*	Reverse primer for <i>fp25k</i> amplification for dsRNA synthesis, T7 promoter specific sequence is underlined; utilised for <i>fp25k</i> sequencing	TTAATACGACTCACTATAGGGGGCTAGAAT ACGACAATCTCACAGAC	8
RDP1079*	Forward primer, binds within <i>chit</i> orf	CAACTGACTTGAACGTCGAC	3
<i>Sfactin</i> -1	Forward primer for <i>Sf21</i> actin qPCR (Allen, 2006)	CGAGGCCCGAGAGCAAGAGAG	3
<i>Sfactin</i> -2	Reverse primer for <i>Sf21</i> actin qPCR (Allen, 2006)	CGCACTGGACGAGAGACACC	4

*Primers were provided by R. D. Possee (OET Ltd.).

+Final primer pair combinations used for multiplex QPCR (Chapter 4)

2.1.7 Antibodies

Primary and secondary antibodies for western blotting (WB; Table 2.8, 2.9) were stored at 4°C or -20°C, respectively. They were diluted into PBS-Tween milk powder (PBS-TMP; Table 2.10) to the appropriate concentration when required.

Table 2.7 Primary antibodies

Antibody	Species	Solvent	Source	Reference
Anti-Cath	Rabbit polyclonal	1:500 (WB)	IVRG	(Saville <i>et al.</i> , 2002)
Anti-ChitA	Guinea pig polyclonal	1:1,000 (WB)	IVRG	(Hawtin <i>et al.</i> , 1995)
Anti-c-MYC (9E10)	Mouse monoclonal	1:500 (WB)	Santa Cruz Biotechnology, Inc.	-
Anti-Histidine tagged	Mouse monoclonal	1:1,000 (WB)	Bio-Rad AbD Serotec Limited	-
Anti-LEF-2	Rabbit polyclonal	1:500 (WB)	GeneScript Biotech. Corporation	This thesis
Anti-P10	Rabbit polyclonal	1:500 (WB)	GeneScript Biotech. Corporation	(Patmanidi <i>et al.</i> , 2003)

Table 2.8 Secondary antibodies

Antibody	Species	Solvent	Source
Anti guinea pig Immunoglobulin G (IgG, Alkaline Phosphatase)	Goat	1:10,000 (WB)	Sigma-Aldrich
Anti mouse IgG (Alkaline Phosphatase)	Goat	1:30,000 (WB)	Sigma-Aldrich
Anti rabbit IgG (Alkaline Phosphatase)	Goat	1:30,000 (WB)	Sigma-Aldrich

2.1.8 Enzymes

Restriction endonucleases, phosphatase, T4 DNA ligase, Taq polymerase and the Q5® High-Fidelity DNA polymerase were supplied from New England Biolabs® Inc. (NEB® Inc.) or Promega. Reactions were performed according to the manufacturer's recommendations.

2.1.9 General reagents and chemicals

General reagents and chemicals were purchased from BDH Chemicals Ltd., Fermentas, Fisher Chemicals, Invitrogen™, NEB® Inc., Sigma-Aldrich, Thermo Fisher Scientific, Promega or VWR.

2.1.10 Buffers and solutions

If not indicated differently, Milli-Q ultrapure water (>18 megohm) was used for preparation of buffers and solutions (Table 2.10). Sterilisation was performed either by autoclaving at 15 pounds per square inch for 15 min or filtration through a 0.45 or 0.2 µm filter.

Table 2.9 Buffers and solutions

Name	Composition
Alkaline Phosphatase	100 mM Tris-HCl pH 9.2, 100 mM NaCl, 5 mM MgCl ₂
Binding buffer (2x)	1 M LiCl, 20 mM Tris pH 7.5, 2 mM EDTA, 0.5% weight/volu (w/v), 1% (v/v) β-Mercaptoethanol (added fresh)
Coomassie blue staining	10% (v/v) glacial acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R-250
Coomassie destaining buffer	10% (v/v) acetic acid
Coomassie fixation buffer	50% (v/v) methanol, 10% (v/v) Acetic Acid
Inoue transformation buffer	55 mM MnCl ₂ ·4 H ₂ O, 15 mM CaCl ₂ ·2 H ₂ O, 250mM KCl, 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) pH 6.7
Laemmli running buffer (1x) (Laemmli, 1970)	1% me (w/v) SDS, 25 mM Tris, 1.92 M glycine
Lysis buffer "sarkosyl" (5x)	20% (w/v) Sodium lauroyl sarcosinate ("sarkosyl"), 10 mM Tris-HCl pH 7.8, 10 mM EDTA
Phosphate-buffered saline (PBS, 1x)	13.7 mM NaCl, 0.27 mM KCl, 0.8 mM Na ₂ HPO ₄ , 0.2 mM KH ₂ PO ₄
PBS-Tween (PBS-T)	PBS, 0.1% (v/v) Tween
PBS-T milk powder (PBS-TMP)	PBS, 0.1% (v/v) Tween, 5% (w/v) milk powder
Sample buffer (6x)	5x TAE, 25% sucrose, 0.5% (w/v) SDS, 0.1% (w/v) bromophenol blue
SDS loading buffer (6x)	25 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 0.05-0.5% (w/v) bromphenol blue, 25% (v/v) β-Mercaptoethanol (added fresh)
Super optimal broth (SOB) medium	2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM Mg ₂ Cl pH 7.0
SOB with catabolite repression (SOC) medium	SOB medium, 20 mM glucose
Tris-acetate (TAE) buffer (1x)	40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA pH 8.0
TE buffer (1x)	100 mM Tris-HCl, 1 mM EDTA pH 8.0
Towbin buffer	25 mM Tris-HCl, 192 mM glycine pH 8.3, 20% (v/v) methanol
Washing buffer (1x)	150 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 1% (v/v) β-Mercaptoethanol (added fresh)

2.2 Virus methods

If not indicated differently, virus work was performed in a sterile environment using aseptic techniques. All virus methods including virus amplification, small-scale virus infection of insect cells, generation of recombinant viruses by co-transfection of insect cells, virus titration by plaque assay, plaque purification, purification of budded viruses from cell culture medium and purification of genomic DNA from baculovirus budded viruses was performed as described previously (King and Possee, 1992). Virus titration by BaculoQUANT™ qPCR was performed according to manufacturer's instructions (Hitchman *et al.*, 2007; OET Ltd.). Infected cellular DNA via column extraction was purified using DNeasy® Blood and Tissue (Qiagen®) as described by the supplier's instructions.

2.3 Bacterial methods

If not indicated differently, all bacterial work was performed in a sterile environment using aseptic techniques.

2.3.1 Generation and transformation of competent bacterial cells

Chemically or physically competent cells were either generated for the heat-shock method or electroporation, respectively (as described in Sambrook and Russel, 2006).

2.3.2 Red®/ET® Recombination

Homologous recombination by Red®/ET® was carried out according to the manufacturer's instructions (Gene Bridges GmbH).

2.3.3 Small scale plasmid purification from bacteria via column extraction

Plasmid DNA from bacterial cultures was purified using DNprep Spin Miniprep kit (Qiagen®) as described by the manufacturer's instructions.

2.4 Molecular biological methods

2.4.1 Restriction enzyme digestion

Restriction enzyme digestion of DNA was performed as recommended by the manufacturer (NEB® Inc.).

2.4.2 Dephosphorylation of DNA

For molecular cloning, the 5' ends of previous digested DNA with restriction enzymes was dephosphorylated using Antarctic Phosphatase (NEB® Inc.) according to the supplier's instruction.

2.4.3 Ligation of DNA

For the ligation of complementary ends of DNA, T4 DNA ligase was utilised (NEB® Inc.) according to the manufacturer's recommendation.

2.4.4 Agarose gel electrophoresis

For the separation of DNA or RNA according to its size, 0.6-2% (w/v) agarose gel electrophoresis was performed as described in Sambrook and Russel, 2006. Gel images were acquired by ChemiDoc™ imaging system (Bio-Rad Laboratories, Inc.).

2.4.5 DNA purification by agarose gel electrophoresis

For DNA purification by agarose gel electrophoresis the designated band was excised using QIAquick gel extraction kit (Qiagen®) as recommended by the supplier.

2.4.6 Polymerase chain reaction (PCR)

DNA amplification was performed using PCR. For cloning purposes the proofreading Q5® High-Fidelity DNA polymerase and for quantitative analysis the Taq DNA polymerase (both NEB® Inc.) were utilised. Reactions were performed according to supplier's instructions and as described in Sambrook and Russel, 2006.

Amplified DNA for cloning purposed was purified using QIAquick PCR purification kit (Qiagen®) according to supplier's instructions.

2.4.7 Site-directed mutagenesis

Site-directed mutagenesis was performed using Q5® Site-directed mutagenesis kit (NEB® Inc.) by following the manufacturer's instructions.

2.4.8 DNA sequencing

DNA sequencing was outsourced and performed by Source BioScience plc (Oxford, UK) using Sanger sequencing. Sequencing data were analysed by using SnapGene® software (version 4.1.7).

2.4.9 RNA sequencing (RNA-seq)

Total RNA from virus or mock-infected cells were isolated by TRIzol® (Invitrogen™) reagent according to the supplier's recommendations. Strand-specific library generation was performed as previously described (Zhong *et al.*, 2011; Chen *et al.*, 2013).

2.5 Biochemical methods

2.5.1 Polyacrylamide gel electrophoresis (SDS-PAGE) for protein analysis, Coomassie Brilliant Blue staining and immuno-(western) blotting

Protein separation was performed by SDS-PAGE (Sambrook and Russel, 2006) and following supplier's information (Bio-Rad Laboratories, Inc.). Proteins were visualised either by Coomassie Brilliant Blue staining or by immunoblotting against the protein of interest as described in Sambrook and Russel, 2006. Images were acquired by ChemiDoc™ imaging system (Bio-Rad Laboratories, Inc.).

2.6 Bioinformatics

Most of the bioinformatic analysis undertaken in this thesis was performed in collaboration with the lab group of Z. Fei (BTI, Ithaca, USA). Filtering, mapping to reference genomes and reads per kilobase per million mapped reads (RPKM) value calculations of raw RNA-seq reads was performed by K. Bao (BTI) as described previously (Chen *et al.*, 2013).

2.7 Statistics

Statistical analysis was performed using Microsoft® Office Excel 2010 and Statistical Package for the Social Sciences (SPSS) v25 (IBM). Graphs were developed by Microsoft® Office Excel 2010 and Adobe® Illustrator CS6.

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Chapter Three:

Involvement of AcMNPV cathepsin in P10 processing and virus-induced cell lysis

3.1 Introduction

The main aim of this thesis was to analyse gene products that are involved in the transition between the viral late and the very late phase of viral infection. The late phase of expression is characterised by BV production, and genes encoding structural proteins such as the major capsid protein VP39 or the DNA condensing protein P6.9 or the viral enzymes ChitA and Cath are expressed (Tweeten *et al.*, 1980; Wilson *et al.*, 1987; Thiem and Miller, 1989b; Hawtin *et al.*, 1995; Slack *et al.*, 1995). Later, in the very late phase of infection nucleocapsids become occluded into polyhedra, which are released upon cell lysis. A characteristic of this phase is the hyperexpression of the two viral genes *polh* and *p10* (Smith *et al.*, 1983c; Pennock *et al.*, 1984; Vlak *et al.*, 1988).

During the analysis of protein synthesis in virus-infected *Sf* cells on a protein gel it was noticed that below P10 a lower molecular weight band appeared very late during the viral replication cycle. This may indicate a possible cleavage of P10 (Weyer *et al.*, 1990). Therefore, before focusing on the main aim of the project it was important to examine the possible role of a P10 cleavage in AcMNPV infection.

The precise role of the non-structural P10 is still unknown. It localises in both the cytoplasm and nuclei of virus-infected cells and is required in different steps towards the end of the viral replication cycle. One potential role of P10 is its implication in nuclear disintegration during cell lysis. Insect cells infected with a *p10* deletion virus did not undergo nuclear lysis (Williams *et al.*, 1989). Thus, it was investigated whether processing of P10 was important for cell lysis to occur.

Other unpublished studies have shown that the observed lower molecular weight band might be a consequence of P10 processing by Cath (R. D. Possee, personal communication). Both Cath and ChitA are viral enzymes that are required for liquefaction of the viral hosts *in vivo*. Insects infected with viruses that either lack the coding sequence of the late genes *cath* or *chitA* or both did not liquefy (Slack *et al.*, 1995; Hawtin *et al.*, 1997). More importantly, Cath was previously associated with a role during cell lysis (Hom *et al.*, 2002). Therefore, it was questioned if the late proteins Cath and/or ChitA had an effect on gene expression of the subsequent very late phase of infection and played a role during the processing of P10. For this reason, an AcMNPV *cath* (Ac Δ cath) deletion virus was generated.

The simultaneous deletion of the adjacent and antiparallel expressed *cath* and *chitA* protects both inter- and intracellular secreted proteins from degradation in the context of recombinant protein expression (Kaba *et al.*, 2004). Even deletion of *chitA* alone has beneficial effects on recombinant protein expression as it reduces protein accumulation

within the ER without affecting viral replication *in vitro* (Possee *et al.*, 1999). Hence, to confirm that the newly generated Ac Δ cath acted *in vitro* as previously described, the *polh* sequence was reinserted into the bacmid (Ac Δ cath^{polh+}) and its effect on virus-induced cell lysis was analysed.

3.2 Generation and verification of Ac Δ cath and Ac Δ cath^{polh+}

Generation of Ac Δ cath was performed by Red[®]/ET[®] recombination using an AcMNPV bacmid in *E. coli* cells resulting in the replacement of the *cath* coding sequence with a *neo*-expressing cassette (Fig. 3.1; Introduction 1.7).

The successful modification of Ac Δ cath DNA was verified by PCR and after the generation of an Ac Δ cath P1 stock (>10⁸ pfu/ml) by immunoblotting against Cath of virus-infected *Sf9* cells (data not shown).

Moreover, to investigate whether the deletion of the *cath* orf resulted in virus-induced cell lysis, the *polh* coding sequence was reinserted into Ac Δ cath by co-transfection of the bacmid DNA and pAcEI-I (kindly provided by R. D. Possee) encoding for AcMNPV polyhedrin. This was followed by four rounds of plaque purification using polyhedra as a marker and the subsequent amplification of Ac Δ cath^{polh+} generating a P1 stock with a high infectivity titre (>10⁸ pfu/ml). Furthermore, the successful insertion of *polh* was verified by PCR (data not shown).

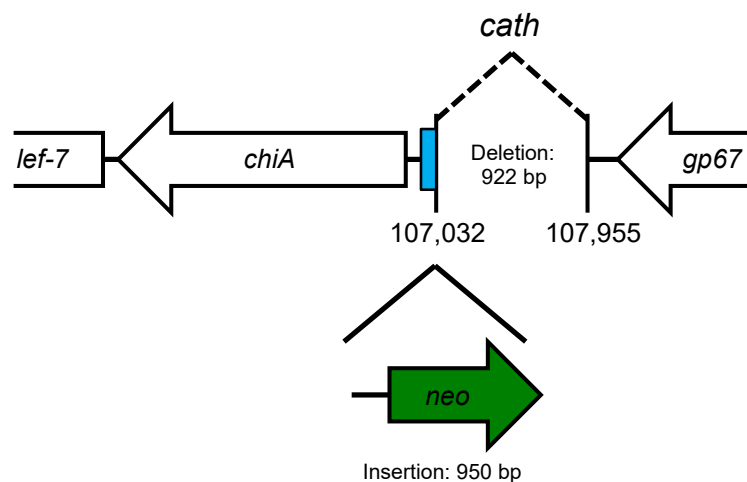


Figure 3.1 Genomic organisation of recombinant Ac Δ cath

The AcMNPV *cath* orf (relative genome position 107,032-107,955) was exchanged with the *neo* encoding nucleotide sequence by Red[®]/ET[®] homologous recombination in *E. coli*.

3.3 The effect of $\text{Ac}\Delta\text{cath}^{\text{polh}+}$ on cell lysis

When *Sf9* cells were infected with AcMNPV, cells underwent minimal cell lysis (7 d p.i., Fig. 3.2). Only a few free polyhedra could be observed and the nucleus of virus-infected cells was still surrounded by cytoplasm. However, in *T. ni* Hi5 cells infection with AcMNPV resulted in extensive cell lysis and release of polyhedra from the nuclei (Fig. 3.3B). On the contrary, when *T. ni* Hi5 cells were infected with $\text{Ac}\Delta\text{p10.lacZ}$, polyhedra were not released into the cell culture medium and remained inside the nuclei of infected cells (Fig. 3.3C). However, the cytoplasm surrounding the virus-infected cells had started to disappear confirming the role of P10 in nuclear disintegration but not plasma membrane disruption.

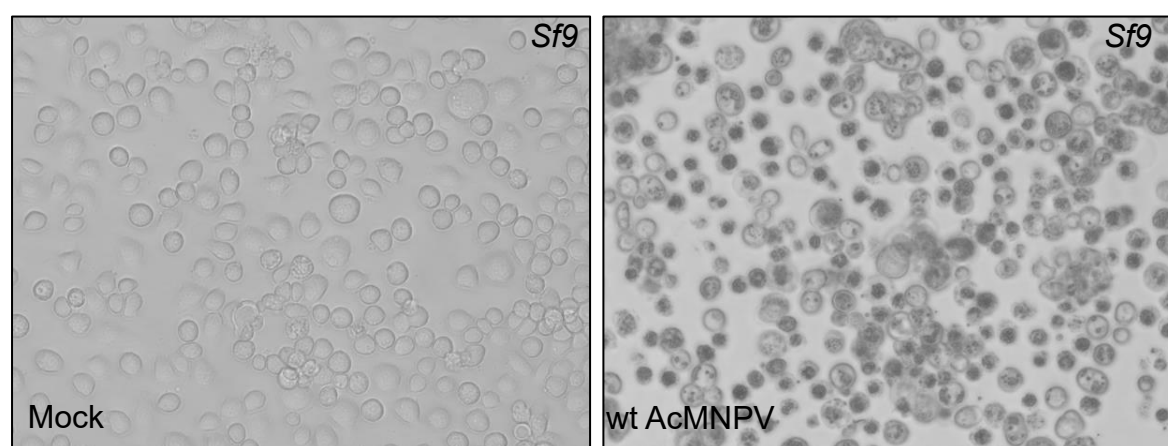


Figure 3.2 The effect of AcMNPV on cell lysis in *Sf9* cells

Sf9 cells were infected with AcMNPV wild-type (wt) at MOI 3 or mock-infected and analysed at 7 d p.i. using light microscope Zeiss Axiovert 135 (100x) for the effect of AcMNPV on cell lysis.

When *T. ni* Hi5 cells were infected with $\text{Ac}\Delta\text{cath}^{\text{polh}+}$ no free polyhedra in the cell culture medium were observed (Fig. 3.3D). The polyhedra remained in the enlarged nuclei of the virus-infected cells. As in $\text{Ac}\Delta\text{p10.lacZ}$ -infected *T. ni* Hi5 cells, cell lysis had been induced after $\text{Ac}\Delta\text{cath}^{\text{polh}+}$ infection but the nuclei were not disintegrated (Fig. 3.3D; Raza, 2013).

3.4 The effect of $\text{Ac}\Delta\text{chit}$ on cell lysis

As the knock-out of *cath* and *chitA* from the AcMNPV genome resulted in a similar phenotype during the liquefaction of the viral host (Slack *et al.*, 1995; Hawtin *et al.*, 1997), the effect of a *chitA* knock-out on cell lysis was investigated. However, a similar picture as in wt-infected *T. ni* Hi5 cells was present when cells were infected with $\text{Ac}\Delta\text{chitA}$. Polyhedra were released into the cell culture medium (Fig. 3.3E). It may be hypothesised that $\text{Ac}\Delta\text{chitA}$ induced cell lysis in *T. ni* Hi5 was delayed compared to AcMNPV-infected cells as there may be fewer polyhedra present in the cell culture medium of cells infected with the recombinant *chitA* deletion virus.

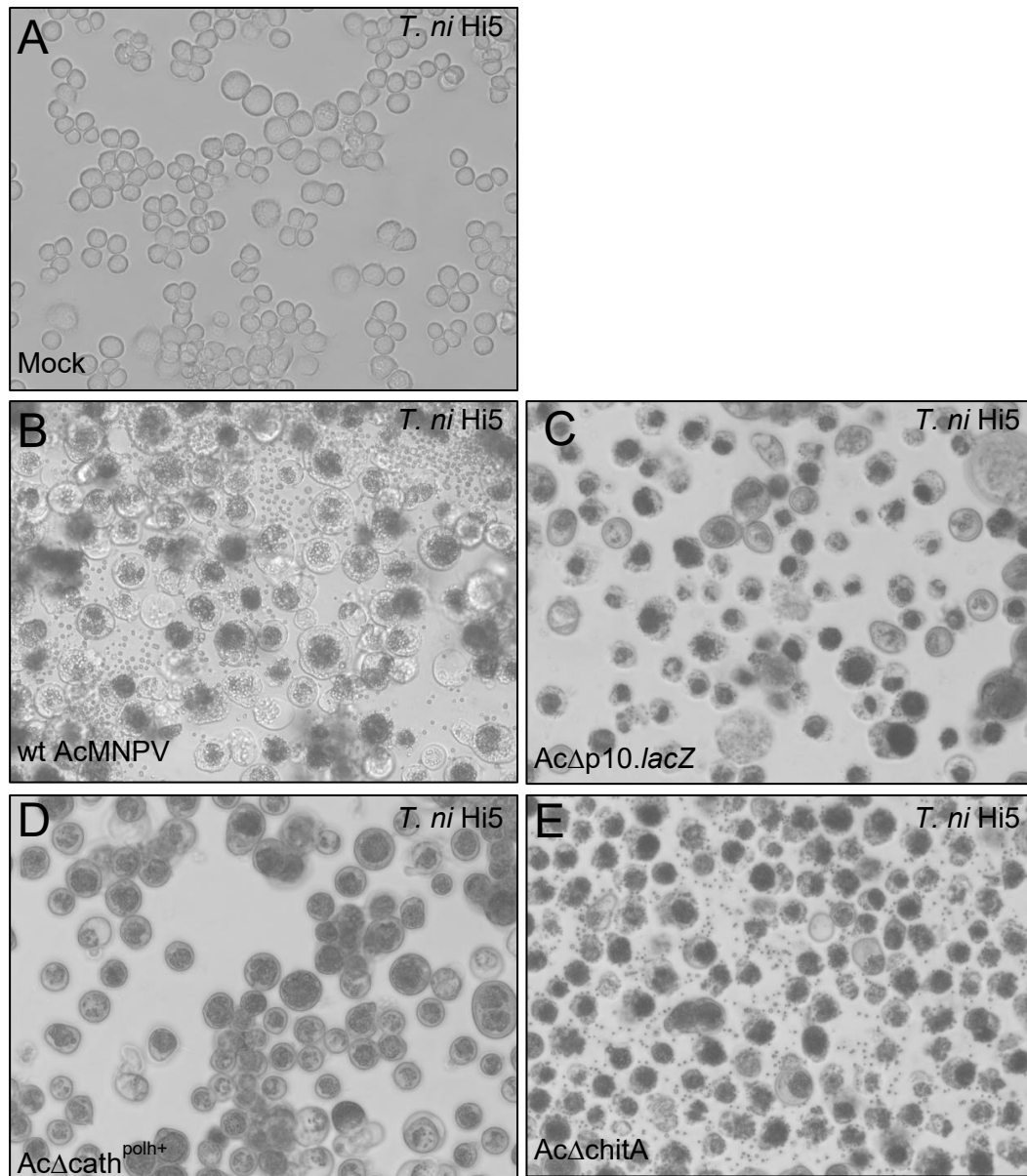


Figure 3.3 The effect of *p10* cathepsin or chitinase A deletion on AcMNPV-induced cell lysis in *T. ni* Hi5 cells

T. ni Hi5 were A) mock-infected or infected with either B) AcMNPV wild-type (wt), C) AcΔ*p10.lacZ*, D) AcΔ*cath^{polh+}* or E) AcΔ*chitA* at MOI 3 and analysed at 7 d p.i. using light microscope Zeiss Axiovert 135 (100x) for the effect of virus gene deletion on cell lysis.

3.5 P10 processing

To investigate the potential cleavage of P10, wt-infected insect cells harvested over a period of 96 h were analysed by a protein gel. The P10 was detectable from 48 h p.i. by either Coomassie staining or immunoblotting against P10 antisera when either *Sf9* or *T. ni* Hi5 cells were infected with AcMNPV. It accumulated to very high levels until the end of the viral replication cycle at 96 h p.i. (Fig. 3.4). Moreover, the accumulation of P10 appeared to be higher in *T. ni* Hi5 than in *Sf9* cells at either time point p. i. (Fig. 3.4). At the same time point as P10 was first detectable, a lower molecular weight protein appeared on the protein gel under P10 for both insect cell lines analysed (Fig. 3.4). The

higher levels of the lower molecular weight protein present at the end of the viral replication cycle, the less P10 was observed. Furthermore, this low molecular weight band was absent when cells were infected with a *p10* deletion virus (*AcΔp10.lacZ*, kindly provided by L. P. Graves) suggesting that this protein may present a cleaved form of P10. However, immunoblotting using a P10 antibody that was directed against the P10 C-terminus did not reveal any second, lower molecular weight band suggesting the lower molecular weight band may represent the P10 N-terminus (Fig. 3.4, 3.5A).

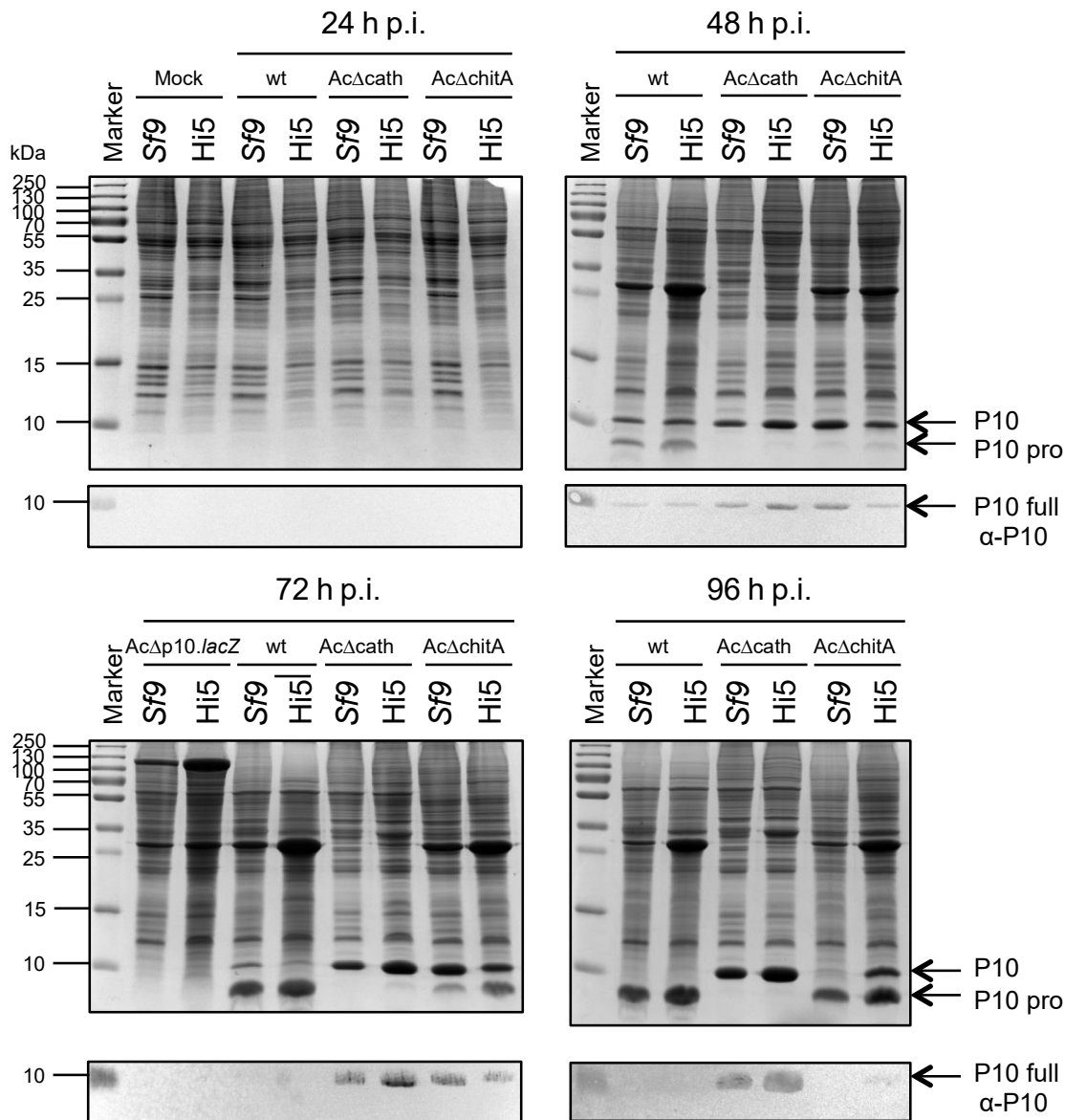


Figure 3.4 P10 time course of virus-infected *Sf9* and *T. ni* Hi5 cells

Sf9 and *T. ni* Hi5 (Hi5) cells were infected with AcMNPV wild-type (wt), *AcΔcath* or *AcΔchitA* at MOI 3 to analyse effects on P10 processing. As negative controls, cells were either mock-infected or infected with a *p10* deletion mutant (*AcΔp10.lacZ*). Virus-infected cells were harvested every 24 h over a period of 96 h. Proteins from cell lysates were separated by a 15% SDS-PAGE and analysed by either Coomassie staining or anti-P10 immunoblotting after protein transfer onto a nitrocellulose membrane. Arrows highlight the full length (P10 or P10 full) and possible processed form of P10 (P10 pro).

To confirm that the lower molecular weight protein was indeed a processed form of P10 and might represent the P10 N-terminus (as it was not detected by the C-terminal-specific P10 antibody), *T. ni* Hi5 cells were infected with a recombinant virus that was 6xHis-tagged at the P10 N-terminus (AcHis.P10, kindly provided by L. P. Graves; Fig. 3.5A). The AcHis.P10-infected cells accumulated the lower molecular weight protein at the end of the viral replication cycle (120 h p.i.; Fig. 3.5B). Immunoblotting against His revealed two distinct bands at the very same time p.i., further indicating that the lower molecular weight band might be a processed form of P10.

A AcMNPV P10:



AcHis.P10:

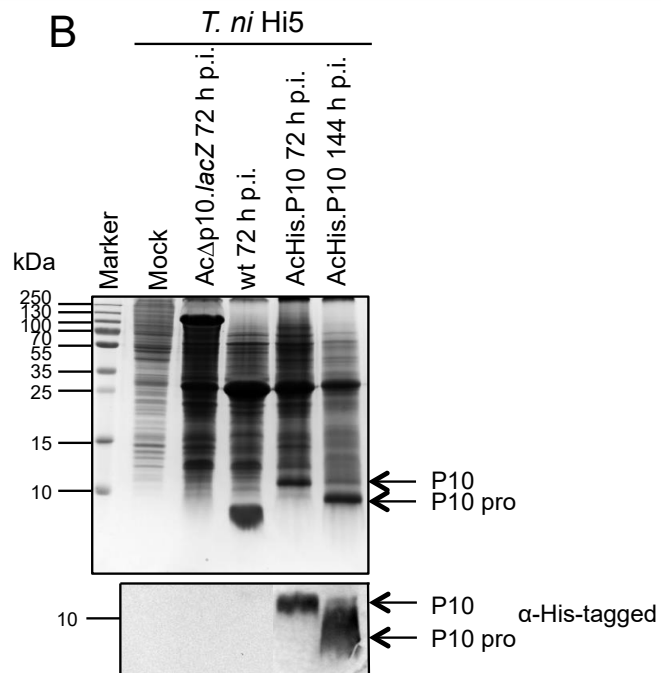


Figure 3.5 Verification of P10 processing by AcHis.P10 infection in *T. ni* Hi5 cells

A) The P10 antibody (α-P10) was directed against the last 15 amino acids at the P10 C-terminus, whereas the N-terminus of AcHis.P10 was 6x His-tagged. P10 structural domains: coiled-coil (green), proline-rich (pink) and basic (blue) B) *T. ni* Hi5 cells were infected with AcHis.p10 at MOI 3. As a positive control cells were infected with AcMNPV wild-type (wt) and as negative controls cells were either mock-infected or infected with a *p10* deletion mutant (AcΔp10.lacZ). Virus infected cells were harvested either at 72 h p.i. or at 144 h p.i. (6 d p.i.). Proteins were separated by a 15% SDS-PAGE and analysed by either Coomassie staining or anti-His immunoblotting after protein blotting onto a nitrocellulose membrane. Arrows highlight the full length and processed form of P10 (P10 pro).

As the knock-out of either *p10* and *cath* resulted in the same phenotype of no nuclear disintegration after virus infection of *T. ni* Hi5 cells (Fig. 3.3C, D) the effect of the protease Cath on P10 processing was investigated in more detail. Moreover, as often the viral

enzymes Cath and ChitA act together (e.g. liquefaction of virus-infected host cadaver; Hawtin *et al.*, 1995), the effect of ChitA on the potential P10 cleavage was determined in addition. Therefore, insect cells were infected with either Ac Δ cath or Ac Δ chitA. Protein analysis of the infected cell lysates revealed that P10 had accumulated from 48 h p.i. until 96 h p.i., which was comparable to AcMNPV-infected cells in both cell lines *Sf9* and *T. ni* Hi5 (Fig. 3.4). However, when the coding region of *cath* was deleted no lower molecular protein was detectable on a protein gel at either time point p.i. Whereas, when the *chitA* orf was deleted, the accumulation of the same protein was delayed by ca. 24 h. This may indicate that the appearance of the low molecular weight protein, which possibly represented a processed form of P10 is dependent on *cath* and to some point on *chitA* expression. Unfortunately, the P10 antibody only accumulated to a faint band after western blotting of Ac Δ chitA-infected *T. ni* Hi5 cell lysates from 96 h p.i. although full length P10 did accumulate on the SDS-PAGE as determined by Coomassie staining (Fig. 3.4). It might be that the protein transfer was not successful onto the nitrocellulose membrane.

To identify potential P10 cleavage sites, *in silico* analysis of the AcMNPV P10 amino acid sequence was performed using the Protease Substrate Specificity Cleavage Site (PROSPER) software (Song *et al.*, 2012). Analysis revealed five potential cathepsin cleavage sites (Fig. 3.6). In total, four potential cleavage sites for cathepsin K and one for cathepsin G were predicted, which were present inside or just after the P10 N-terminal coiled-coil region.

AcMNPV P10 with potential cathepsin cleavage sites:

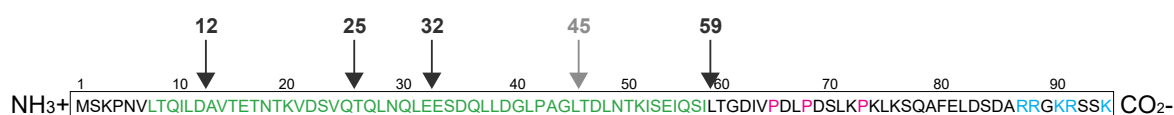


Figure 3.6 Potential P10 cleavage sites for cathepsin

Schematics of AcMNPV P10 amino acids with potential cathepsin cleavage sites as predicted by PROSPER software (Song *et al.*, 2012). Dark grey arrows: cathepsin K and light grey arrow: cathepsin G potential cleavage sites; The number above the arrows corresponds to the P10 amino acid number after which the potential cathepsin cleavage could occur. P10 structural domains: coiled-coil (green), proline-rich (pink) and basic (blue).

To further examine if P10 was processed during virus infection and was not an artefact of virus-infected cell lysate preparation for SDS-PAGE, the same experiment (as Fig. 3.4) was repeated but this time a protease inhibitor (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche) was included during the extraction of samples. Furthermore, all steps during sample extraction and preparation were performed on ice or at 4°C.

Infection of neither *Sf9* nor *T. ni* Hi5 cells with either AcMNPV, Ac Δ cath or Ac Δ chitA led to

the accumulation of the lower molecular weight P10 when the protein inhibitor was present during the harvesting process between 24-96 h p.i. (Fig. 3.7). This suggested that processing of intracellular P10 was an artefact probably caused by the viral proteinase Cath during sample harvesting and preparation. For this reason, a protease inhibitor was always added during the harvesting process of virus-infected cells in all following experiments in this thesis.

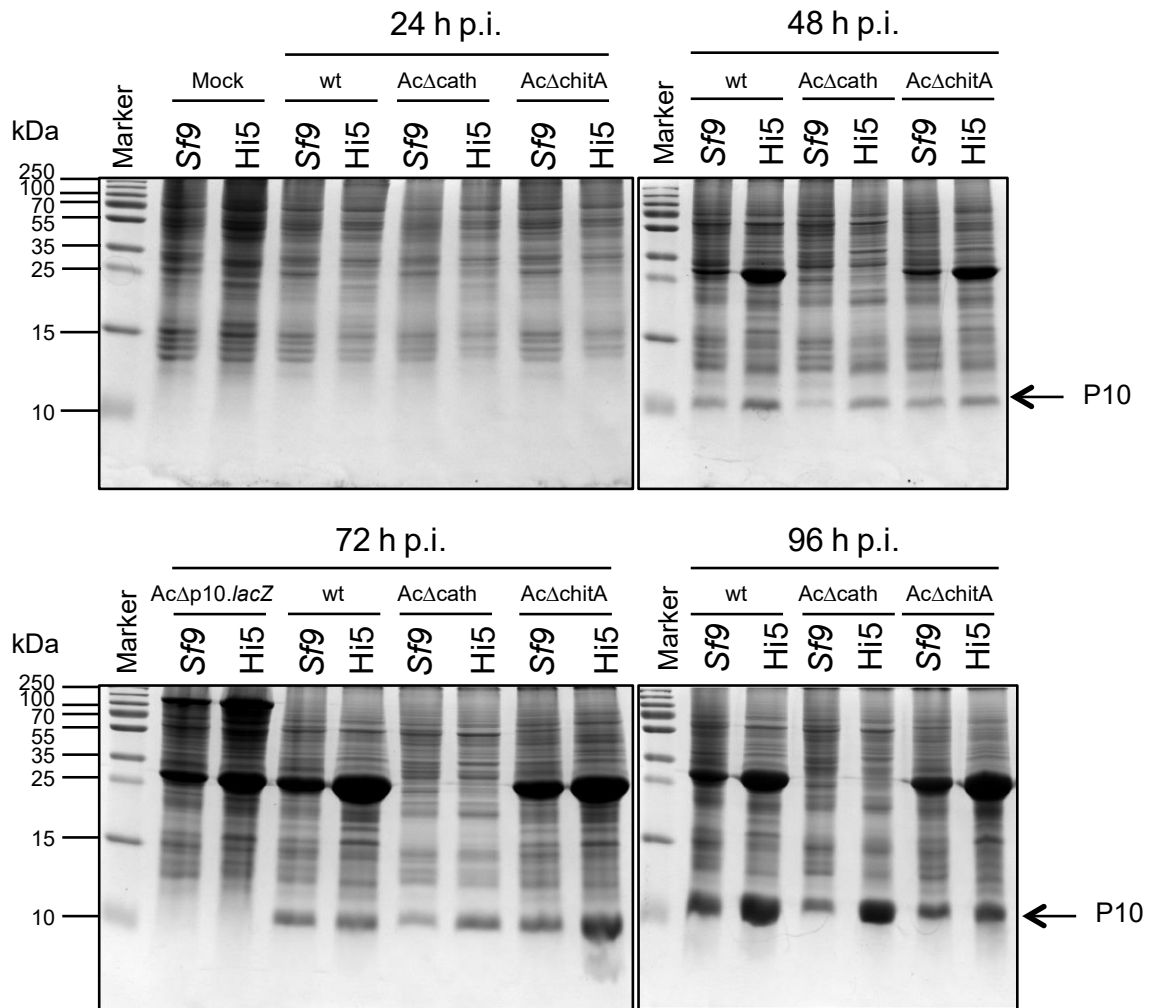


Figure 3.7 P10 time course with the addition of a protease inhibitor during the harvesting process in virus-infected Sf9 and *T. ni* Hi5 cells

Sf9 and *T. ni* Hi5 (Hi5) cells were infected with AcMNPV wild-type (wt), AcΔcath or AcΔchitA at MOI 3 to analyse their effect on P10 processing. As negative controls cells were either mock-infected or infected with a *p10* deletion mutant (AcΔp10.lacZ). Infected cells were harvested every 24 h over a period of 96 h p.i. A protease inhibitor was added during the harvesting process and all steps were performed at 4°C. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. The arrow highlights the full-length form of P10.

However, both P10 and Cath are spatially separated inside the cell. While P10 localises in the cytoplasm and the nucleus, Cath is present in the ER of virus-infected cells (van der Wilk *et al.*, 1987; Hodgson *et al.*, 2009). Moreover, pro-Cath remains mainly inactive until cells death, when it is processed into the catalytically active Cath. Furthermore, pro-Cath is associated with dead cells in the cell culture medium of virus-infected cells and was

associated with cell lysis (Hom *et al.*, 2002). Hence, it was questioned, whether P10 processing by Cath may occur in the cell culture medium after cell lysis has started, the plasma membrane was disrupted and Cath was released.

To investigate the potential role of P10 processing for nuclear disintegration in more detail, the cell culture media of wt, $\Delta\text{cath}^{\text{polh}+}$, ΔchitA or $\Delta\text{p10.lacZ}$ -infected *Sf9* or *T. ni* Hi5 cells were harvested at either 7 or 14 d p.i. in the presence of a protease inhibitor. Proteins were extracted and purified using StrataClean Resin (Agilent Technologies). In general, more extracellular protein was accumulated in *T. ni* Hi5 than *Sf9* cells (Fig. 3.8), which may be either secreted from the cell or released upon virus-induced cell lysis. In the clarified cell culture medium of wt-infected cells both P10 and the processed form of P10 had accumulated by 7 d p.i., while at 14 d p.i. only the cleaved form of P10 was present (Fig. 3.8). This suggested that P10 processing was not an artefact and could be detected in the cell culture medium after the cells have started to lyse. Interestingly, when cells were infected with $\Delta\text{cath}^{\text{polh}+}$ only full length but no processed P10 accumulated in the cell culture medium at 7 d p.i. Moreover, only a very small amount of cleaved P10 accumulated at 14 d p.i., while the majority of P10 was still unprocessed (Fig. 3.8). This indicated that Cath may be required for P10 cleavage. When cells were infected with ΔchitA processed P10 accumulated in the clarified cell culture medium suggesting that P10 cleavage is dependent on Cath but not ChitA (Fig. 3.8). As expected, cells infected with $\Delta\text{p10.lacZ}$ did not accumulate P10 in the cell culture medium (Fig. 3.8).

To determine if P10 was cleaved intracellularly or in the cell culture medium wt-infected *T. ni* Hi5 cells were incubated in the presence of increasing concentrations of FCS [0-20% (v/v)]. The FCS may hinder extracellular P10 cleavage by providing alternative substrates for the protease. As almost no protein could be detected in the cell culture medium of *Sf9*-infected cells (Fig. 3.8), only *T. ni* Hi5 cells were utilised. At 12 d p.i. the cell culture medium of virus-infected cells was harvested and proteins were extracted as described before. While processed P10 was found to be accumulated in the clarified cell culture medium of wt-infected cells when no or only 5%(v/v) FCS was present, both full length and cleaved P10 were present when virus-infected cells were incubated with $\geq 10\%$ (v/v) FCS (Fig. 3.9). This suggested that P10 is processed in the cell culture medium of virus-infected cells.

To investigate whether P10 could be cleaved by Cath, the presence of Cath in the clarified cell culture medium of virus-infected *T. ni* Hi5 cells was determined (Fig. 3.10). Accumulation of Cath was detectable in the cell culture medium of wt, $\Delta\text{p10.lacZ}$ and

Ac Δ chiA but not in Ac Δ cath^{polh+}-infected *T. ni* Hi5 cells as determined by immunoblotting against Cath. This indicated that potentially extracellular P10 could be processed by Cath.

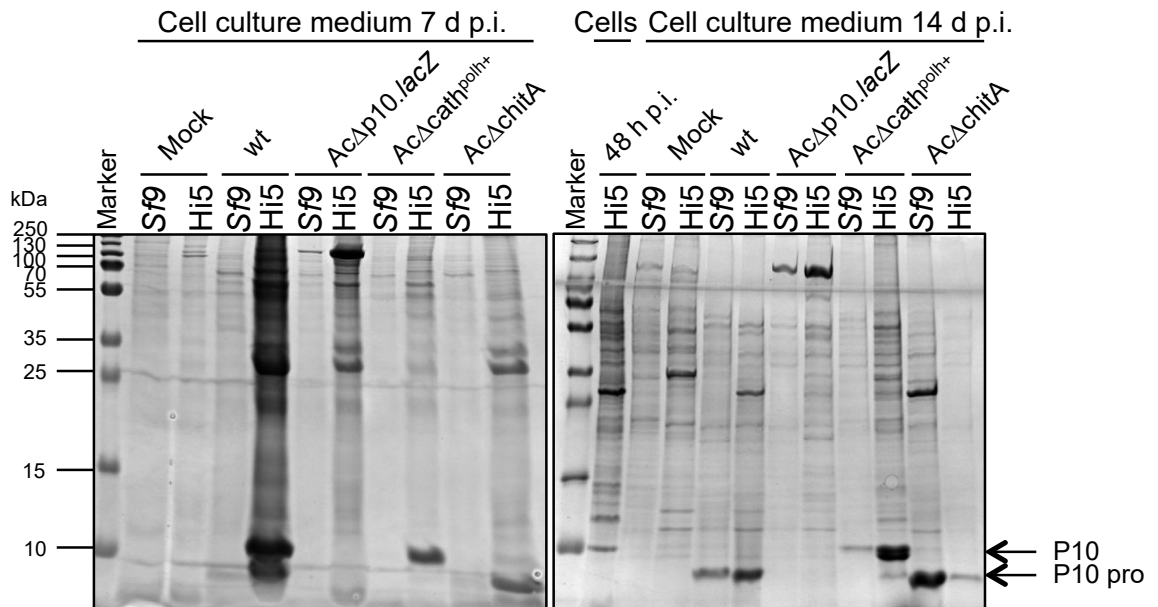


Figure 3.8 P10 processing in the cell culture medium of AcMNPV-infected *T. ni* Hi5 and *Sf9* cells and the effects of cathepsin and chitinase A deletion on protein cleavage

Sf9 and *T. ni* Hi5 (Hi5) cells were infected with AcMNPV wild-type (wt), Ac Δ cath^{polh+} or Ac Δ chitA at MOI 3 to analyse their effect on P10 processing. As negative controls cells were either mock-infected or infected with a *p10* deletion mutant (Ac Δ p10.lacZ). The cell culture medium was harvested either at 7 or 14 d p.i. A protease inhibitor was added during the harvesting process and all steps were performed at 4°C. Cell lysates from wt-infected *T. ni* Hi5 cells (48 h p.i.) were run as a further control. Extracellular proteins were extracted, purified, subsequently separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight the full length and processed form of P10 (P10 pro).

To determine if P10 was indeed processed by Cath and furthermore if processed P10 was essential for cell lysis the clarified cell culture medium from Ac Δ p10.lacZ-infected *T. ni* Hi5 cells was added to Ac Δ cath^{polh+}-infected cells at 6 d p.i. (Fig. 3.11). The cell culture medium of Ac Δ cath^{polh+}-infected *T. ni* Hi5 cells was enriched in full length P10 (Fig. 3.13), while the cell culture medium of Ac Δ p10.lacZ-infected cells contained Cath (Fig. 3.10). Both virus-induced nuclear disintegration and P10 cleavage was analysed 24 h after the addition of the medium (after the addition of Cath). Polyhedra release and therefore nuclear disintegration was neither observed after Ac Δ cath^{polh+} nor Ac Δ p10.lacZ infection (Fig. 3.3C, D). However, an extensive amount of released polyhedra was evident after the cell culture medium of Ac Δ p10.lacZ-infected cells was added to Ac Δ cath^{polh+}-infected cells (Fig. 3.12A). Moreover, the presence of Cath inside the cell culture medium led to processing of P10 indicating that P10 is cleaved by Cath (Fig. 3.13). To confirm that processed P10 induced nuclear disintegration the clarified cell culture medium of Ac Δ p10.lacZ-infected cells was added to Ac Δ cath Δ p10-infected *T. ni* Hi5 cells at 6 d p.i. Hence, as neither of the viruses encoded for P10, it did not accumulate in the cell culture

medium of virus-infected cells (Fig. 3.8, 3.13). But more importantly, *Ac* Δ cath Δ p10-infected *T. ni* Hi5 cells did not release polyhedra when Cath was provided (Fig. 3.12B).

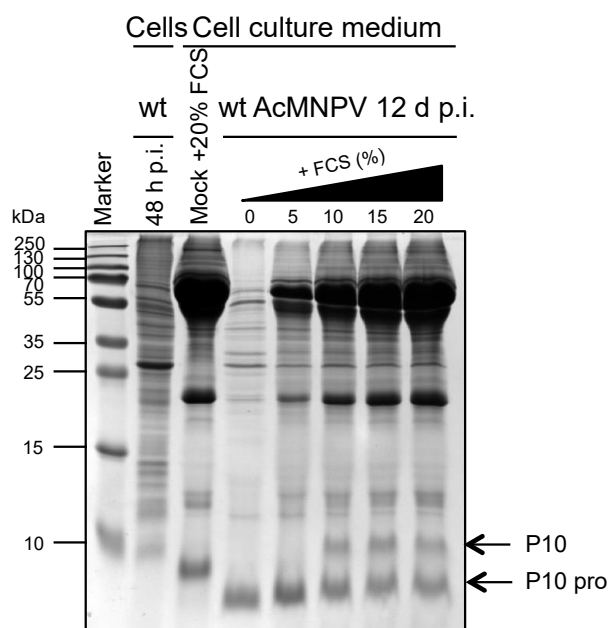


Figure 3.9 P10 is cleaved in the cell culture medium of AcMNPV-infected *T. ni* Hi5 cells

T. ni Hi5 cells were infected with AcMNPV wild-type (wt) at MOI 3 in the presence of FCS [0-20% (v/v)] to analyse if P10 processing occurs in the cell culture medium. The cell culture medium was harvested at 12 d p.i. A protease inhibitor was added during the harvesting process and all steps were performed at 4°C. Cell lysates from wt-infected *T. ni* Hi5 cells (48 h p.i.) were run as a further control. Extracellular proteins were extracted, purified, subsequently separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight the full length and processed form of P10 (P10 pro).

To determine the potential cleavage site within P10 (Fig. 3.6; Table 3.1), the molecular mass of processed P10 was examined using both commercial and homemade lower molecular weight protein markers. However, attempts were unsuccessful as the molecular masses of the low molecular weight protein markers did not agree with the ones of either the routinely used broad molecular weight protein marker or the viral P10 (data not shown). Therefore, attempts to estimate the mass of the processed P10 were not proceed further.

3.6 Discussion

The main aim of this thesis was to determine how both the late and very late phase of gene expression is regulated after AcMNPV infection. However, as it was recognised that the viral very late protein P10 might be processed (Weyer *et al.*, 1990), the objective of this chapter was to determine the function of the possible cleavage of P10 *in vitro*.

The viral P10 is a small protein of about 10 kDa in size and is hyperexpressed very late in virus infected cells (Fig. 3.4; Kuzio *et al.*, 1984). Synthesis of *p10* mRNA occurs from 18 h p.i. and from 24 h p.i. *p10* is together with *polh* the most abundant transcribed viral

mRNA (Kuzio *et al.*, 1984; Chen *et al.*, 2013). On a protein gel P10 accumulation was detected from 48 h p.i. in both *T. ni* Hi5 and *Sf9* virus-infected cells either by Coomassie staining or by immunoblotting against P10 after protein transfer onto a nitrocellulose membrane (Fig. 3.4). Although P10 is hyperexpressed in both cell lines, it accumulated to a higher level in *T. ni* Hi5 cells (Fig. 3.4, 3.7).

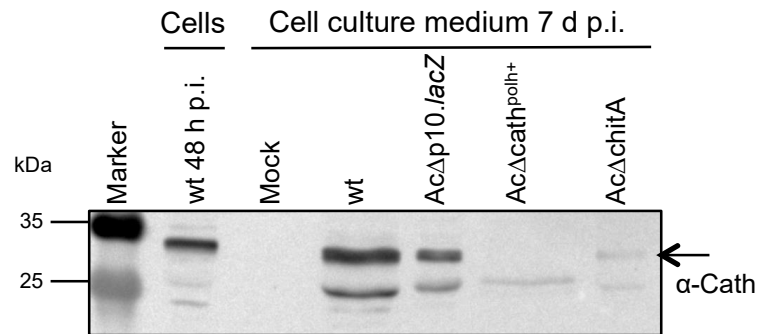


Figure 3.10 Cathepsin is present in the cell culture medium

T. ni Hi5 cells were infected with AcMNPV wild-type (wt), AcΔp10.lacZ or AcΔchitA at MOI 3 to analyse if cathepsin (Cath) is present in the cell culture medium of virus-infected cells. As negative controls cells were either mock-infected or infected with a *cath* deletion mutant (AcΔcath^{polh}⁺). The cell culture medium was harvested at 7 d p.i. A protease inhibitor was added during the harvesting process and all steps were performed at 4°C. Cell lysates from wt-infected *T. ni* Hi5 cells (48 h p.i.) were run as a further control. Extracellular proteins were extracted, purified and subsequently separated by a 15% SDS-PAGE and analysed immunoblotting against Cath. Arrow highlights Cath.

Even the phenotypic observation of AcMNPV-infected cells by light microscopy revealed cell line specific differences between *T. ni* Hi5 cells and *Sf9* cells. While AcMNPV-infected *T. ni* Hi5 cells released large number of polyhedra into the cell culture medium via cell lysis, *Sf9* underwent minimal cell lysis and polyhedra release at 7 d p.i. (Fig. 3.2, 3.3B).

Cell line specific differences in response to AcMNPV infection have been described before. The viral P35 acts as an apoptosis inhibiting protein in the non-permissive *Sf* cell line infected with AcMNPV. Deletion of AcMNPV *p35* and subsequent infection of *Sf* cells almost immediately induces apoptosis. On the contrary, deletion of the *p35* coding sequence in AcMNPV did not affect viral infection in *T. ni* cells (Clem *et al.*, 1991; Hershberger *et al.*, 1992; Griffiths *et al.*, 1999). Another example of a viral protein with a cell line specific is LEF-12. The *lef-12* orf was identified to be essential for viral late gene expression in a transient expression assay in *Sf21* cells. However, it had only stimulatory effects on viral late gene expression in a similar experimental design in *T. ni* (TN368) cells (Rapp *et al.*, 1998).

When cells were further infected with a recombinant virus containing a *p10* deletion, they failed to release polyhedra into the cell culture medium, which was a result of impaired nuclear disintegration (Fig. 3.3C; Williams *et al.*, 1989; van Oers *et al.*, 1993). However, nuclear lysis was not quantified by qualitative approaches such as propidium iodide

staining to evaluate cell viability or by the colorimetric lactate dehydrogenase (LDH) assay, which measures the release of LDH into the cell culture medium after cell damage and is therefore utilised as an apoptosis assay. However, this was done elsewhere (van Oers *et al.*, 1993; Raza, 2013). When either *Sf21* cells (van Oers *et al.*, 1993) or *T. ni* Hi5 cells (Raza, 2013) were infected with a recombinant AcMNPV lacking the entire *p10* coding sequence no significant difference of LDH release was observed compared to wt-infected cells suggesting the same amount of plasma membrane damage.

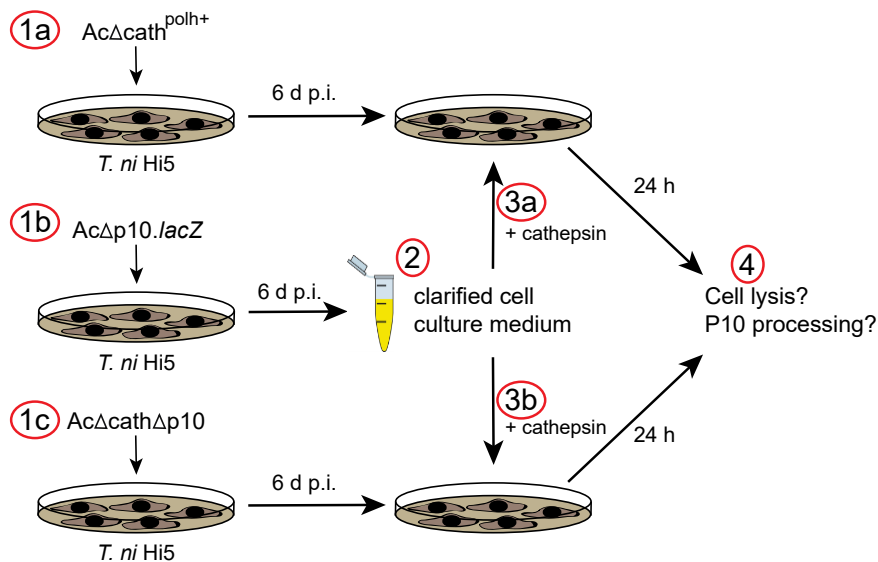


Figure 3.11 Experimental outline to investigate P10 processing by cathepsin for virus-induced cell lysis

T. ni Hi5 cells were infected with either 1a) $\text{Ac}\Delta\text{cath}^{\text{polh}+}$, 1b) $\text{Ac}\Delta\text{p10.lacZ}$ or 1c) $\text{Ac}\Delta\text{cath}\Delta\text{p10}$ at MOI 3. At 6 d p.i. the cell culture medium of $\text{Ac}\Delta\text{p10.lacZ}$ -infected cells was harvested, 2) any cell debris were removed by centrifugation (4,000g, 2min) and added to 3a) $\text{Ac}\Delta\text{cath}^{\text{polh}+}$ or 3b) $\text{Ac}\Delta\text{cath}\Delta\text{p10}$ -infected cells thereby providing cathepsin. 4) After 24 h cell lysis was analysed by light microscopy to determine nuclear disintegration (Fig. 3.12) and cell culture media were subsequently harvested to investigate P10 processing (Fig. 3.13).

Interestingly, when the AcMNPV *p10* coding sequence was replaced by the sequence of SeMNPV insect cells infected with this recombinant virus failed to release polyhedra into the cell culture medium. It was therefore hypothesised that P10 might interact with at least one virus-specific factor (van Oers *et al.*, 1994).

However, by the detection of viral P10 from AcMNPV-infected cell lysates on a polyacrylamide gel, it was further discovered that a lower molecular weight band accumulated very late during the viral replication cycle (from 48 h p.i.; Fig. 3.4; Weyer *et al.*, 1990). Further analysis suggested that this lower molecular weight band was a processed form of P10 and may represent its N-terminus as this band could be resolved after infection with a N-terminally His-tagged P10 AcMNPV after immunoblotting against His but not after western blotting using a P10 antibody, which was directed against the P10 C-terminus (Fig. 3.4, 3.5). Moreover, this lower molecular weight band was absent

when cells were infected with a recombinant *p10* deletion virus (Fig. 3.4). It may be further speculated that the molecular mass of the P10 C-terminus was too low to be visualised by mini protein electrophoresis.

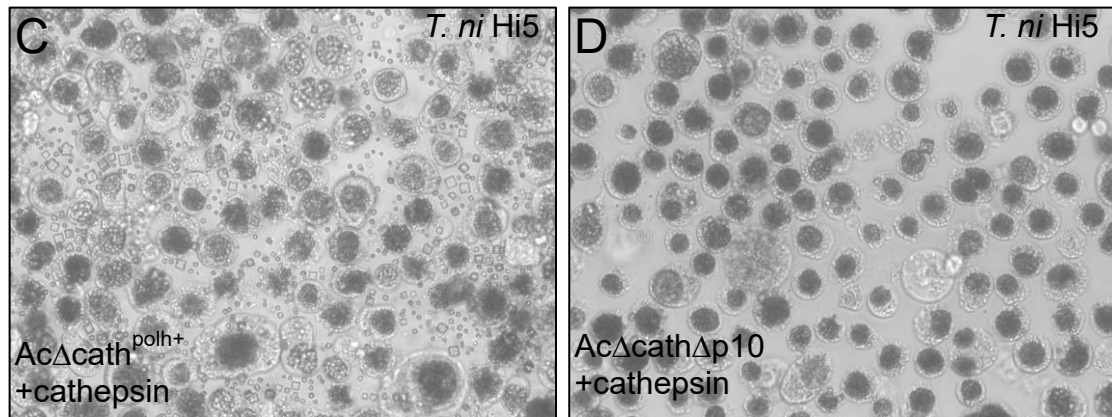


Figure 3.12 P10 processing by cathepsin is required for virus-induced cell lysis.

T. ni Hi5 cells were infected with A) AcΔcath^{polh+} or B) AcΔcathΔp10. Clarified cell culture medium of AcΔp10.*lacZ*-infected *T. ni* Hi5 cells was added to virus-infected cells at 6 d p.i. (+cathepsin) and polyhedra release was analysed 24 h after the addition of the cell culture medium using light microscope Zeiss Axiovert 135 (100x) for the effect of virus gene deletion on cell lysis. Controls are shown in Figure 3.3.

Interestingly, in the case of AcMNPV the viral protease Cath seemed to be required for cleavage of P10. When cells were infected with a recombinant virus lacking the *cath* coding sequence no cleaved P10 could be observed (Fig 3.4). Moreover, when cells were infected with AcΔchitA the appearance of the processed P10 was delayed (Fig. 3.4). This may be because Cath processing is to some point dependent on ChitA (Hom and Volkman, 2000). Nevertheless, the addition of a inhibitor cocktail against a wide range of both serine and cysteine proteases revealed that the lower molecular weight P10 was an artefact possibly dependent on the viral proteinase Cath during the harvesting process. The addition of a protein protease inhibitor completely abolished the appearance of the lower molecular band (Fig. 3.7). The omission of a protease inhibitor during sample preparation already previously resulted in the false assumption that Pro-Cath is rapidly processed after expression in virus-infected cells, although processing does not occur before cell death (Slack *et al.*, 1995; Hom *et al.*, 2002).

In silico analysis revealed five potential cathepsin cleavage sites in P10 (Fig. 3.6). Moreover, as P10 and Cath are spatially separated inside different cellular compartments and pro-Cath was associated with dead cells in the cell culture medium of virus-infected cells (van der Wilk *et al.*, 1987; Hom *et al.*, 2002; Hodgson *et al.*, 2009), it was hypothesised that P10 processing may occur after cell lysis has been induced, the plasma membrane has been damaged and Cath was released.

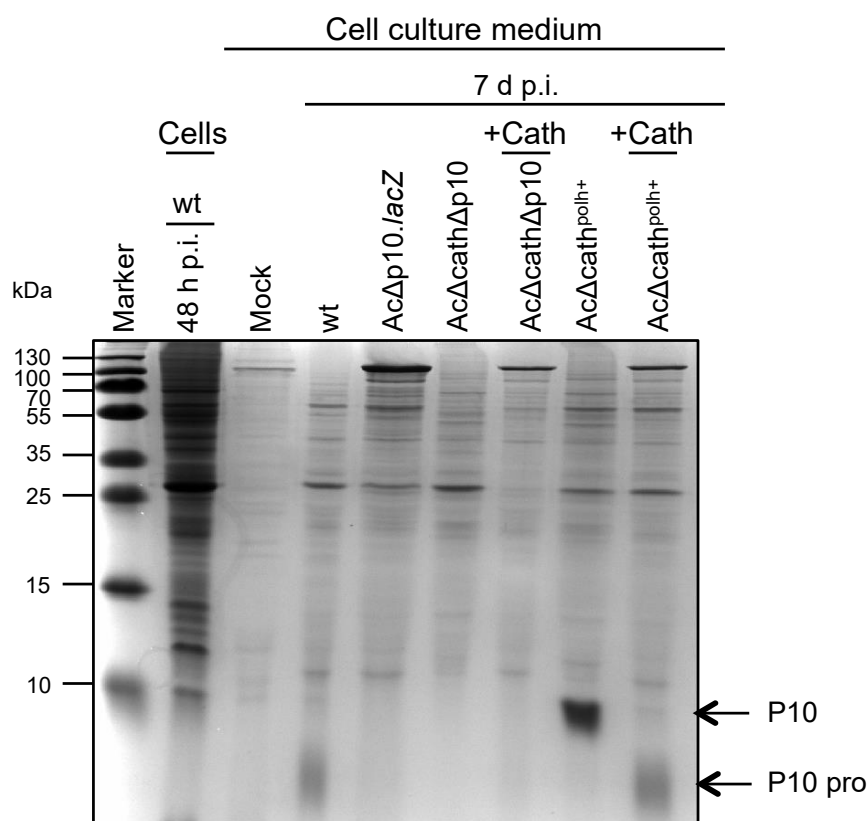


Figure 3.13 AcMNPV P10 is processed by cathepsin

T. ni Hi5 cells were infected with AcMNPV wild-type (wt), AcΔp10./acZ, AcΔcathΔp10 or AcΔcath^{polh+} at MOI 3 to analyse effects on P10 processing. As a negative control cells were mock-infected. Clarified cell culture medium of AcΔp10./acZ was added to AcΔcathΔp10 or AcΔcath^{polh+}-infected cells at 6 d p.i. (+Cath). The cell culture medium was harvested at 7 d p.i. A protease inhibitor was added during the harvesting process and all steps were performed at 4°C. Cell lysates from wt-infected *T. ni* Hi5 cells (48 h p.i.) were run as a further control. Extracellular proteins were extracted, purified, subsequently separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight the full length and processed form of P10 (P10 pro).

Usually, proteins are processed to spatially and temporally regulate their function. One prominent example of a virus that regulates its protein function by cleavage is HIV. The retrovirus HIV-1 expresses a viral protease (PR) that cleaves the HIV Gag-Pol (Gag = group antigens/capsid protein; Pol encodes for: DNA polymerase, integrase/protease, reverse transcriptase) polyprotein after virion budding from the plasma membrane into its functional units Gag and Pol. Without this specific processing the HIV-1 virions would be non-infectious (reviewed by Konvalinka *et al.*, 2015).

Table 3.1 Estimated molecular masses of the predicted processed P10 forms*

Cleavage after P10 amino acid	Estimated mass
59	6.46 kDa
45	4.90 kDa
32	4.49 kDa
25	2.73 kDa
12	2.42 kDa

*Fig. 3.6 for details on predicted P10 cleavage sites; masses predicted by PROSPER software (Song *et al.*, 2012).

Interestingly, processed P10 was accumulated in the cell culture medium of wt-infected cells from 7 d p.i. suggesting that P10 cleavage was not an artefact and occurred after cell lysis had started (Fig. 3.8). Moreover, P10 was not processed when infected with *AcΔcath^{polh+}* indicating that Cath was involved in this event (Fig. 3.8). Together with Cath, ChitA liquefies the insect host cadaver after cell death (Hawtin *et al.*, 1997). Moreover, ChitA proteins may process Pro-Cath into a catalytically active enzyme by acting as a chaperone (Hom and Volkman, 2000; Hodgson *et al.*, 2013), yet cells infected with a *chitA* knock-out mutant did accumulate the potential processed form of P10 in the cell culture medium (Fig 3.8).

Differences in the function of Cath and ChitA were further observed in the relation of nuclear disintegration of cells infected with knock-out versions of those viral enzymes. Whereas cell lysis of *T. ni* Hi5 cells infected with *AcΔchitA* occurred similar to wt-infected cells and released polyhedra into the cell culture medium, *AcΔcath^{polh+}* infected cell did not release any polyhedra suggesting that nuclear lysis was impaired as a cause of *cath* deletion (Fig. 3.3; Raza, 2013).

However, most importantly, Cath specifically cleaved P10 in the cell culture medium of virus-infected cells. Moreover, the cleaved form of P10 was essential for polyhedra release and therefore for nuclear disintegration. If Cath was provided by adding the cell culture medium of *AcΔp10.lacZ* to *AcΔcath^{polh+}*-infected cells at 6 d p.i., polyhedra were released. Furthermore, P10 was processed. When the same cell culture medium was added to *AcΔcathΔp10*-infected cells, polyhedra still remained in the nucleus of infected cells. Therefore, Cath was identified as the virus-specific factor that interacts with P10 for nuclear lysis of virus-infected cells (3.8-3.13; van Oers *et al.*, 1994).

In virus-infected cells, P10 localises in both the nucleus and cytoplasm (Quant-Russell *et al.*, 1987; Volkman and Zaal, 1990; Patmanidi *et al.*, 2003; Carpentier *et al.*, 2008). Nuclear P10 is found in close association with electron dense structures and polyhedra (Summers and Arnott, 1969; Hess and Falcon, 1978). Moreover, the intranuclear P10 is required for polyhedra morphogenesis (Vlak *et al.*, 1988; Williams *et al.*, 1989). When insect cells were infected with a recombinant *p10-lacZ* fusion virus polyhedra were not surrounded by a membrane (Vlak *et al.*, 1988). However, the role of cytoplasmic P10 still remains elusive. It forms two distinct cytoskeletal structures for which it does not require any other viral protein. While thin P10 tubules inside the cytoplasm are formed around 24-48 h p.i., which interact with the microtubule network via the P10 C-terminus, thick rod-like perinuclear tubes are present around the nucleus forming a dome-shaped perinuclear cage (Patmanidi *et al.*, 2003; Carpentier *et al.*, 2008). This structure does not co-localise with microtubules. Furthermore, it is formed later in virus-infected cells and was still

detectable at 96 h p.i. suggesting a role at the very end of the viral replication cycle (Patmanidi *et al.*, 2003; Carpentier *et al.*, 2008; Raza *et al.*, 2017). The P10 perinuclear cage structure stabilises the nucleus by preventing nuclear disruption. Hence, it allows polyhedra maturation by preventing an early release (Carpentier *et al.*, 2008). Eventually the cleavage of perinuclear P10 by Cath initialises nuclear disintegration by a stepwise exhaustion of the host nuclear membrane ultimately leading to rapture and the release of matured polyhedra (Williams *et al.*, 1989).

Unfortunately, it was impossible to determine the molecular mass of the processed P10 (data not shown). An alternative method to identify the P10 cleavage site would be by matrix-assisted laser desorption ionisation-in source decay (MALDI-MS/MS). This is an ideal technique for accessing the position of the truncated form. However, protein identification by MALDI-MS/MS is not possible from protein extraction from SDS-PAGEs and the protein should be provided pure. One option to purify a protein would be by tagging the protein and by pull down assays. However, a P10 protein tag may interfere with its function in nuclear lysis.

Nevertheless, a bioinformatic approach determined the potential P10 cleavage sites by Cath after amino acids 12, 25, 32, 45 or 59, which are either located within the P10 coiled-coil region (amino acids 12, 25, 31, 45) or just after (amino acid 59; Fig. 3.6). Coiled-coil regions are structurally highly organised and usually involved in the oligomerisation of multimeric proteins. The AcMNPV P10 contains seven heptated repeat motifs (van Oers *et al.*, 1993; Wilson *et al.*, 1995). Therefore, it is unlikely that cleavage occurs inside the highly organised coiled-coil domain (reviewed by van Oers *et al.*, 1994). Furthermore, the function of P10 to be required for nuclear disintegration was assigned to its C-terminus (Williams *et al.*, 1989; Chaabihi *et al.*, 1993), more specifically it was mapped by mutagenesis studies to the *p10 BgIII-HindIII* fragments, which encodes for amino acids 52-79 (van Oers *et al.*, 1993). Therefore, we hypothesised that P10 cleavage by Cath occurs between lysine (Lys, L) 59 and threonine (Thr, T) 60. The easiest way to prove this hypothesis right would be by site-directed mutagenesis studies of the predicted P10 cleavage site. However, the successful structural formation of P10, especially the perinuclear structures, in the recombinant virus-infected cells should be verified. This is particularly important as different protein domains have been investigated to play a crucial role in P10 filament formation. When the basic P10 C-terminus was deleted, virus-infected cells did only generate P10 aggregates, while P10 filaments failed to form when cells were infected with a recombinant virus that did not encode for the P10 coiled-coil region (N-terminus; van Oers *et al.*, 1993; Dong *et al.*, 2007). Moreover, phosphorylation of P10 Ser 93 may induce the aggregation of filaments to form the perinuclear tubes (Raza *et al.*, 2017).

3.7 Conclusion

During the analysis of protein synthesis in AcMNPV-infected cells, a protein with lower molecular weight than P10 was observed on a protein gel. As a potential role of P10 is its requirement in nuclear disintegration the involvement of P10 processing in polyhedra release was investigated. It could be shown that the lower molecular weight protein was indeed a processed form of P10 and represented its N-terminus. Moreover, it appears that cleavage of P10 specifically occurred by Cath but does not require CathA. Processing of P10 by Cath occurred in the cell culture medium of AcMNPV-infected insect cells after cell lysis had been induced and Cath was released from the ER. The specific cleavage site could not be determined. However, *in silico* analysis suggested that processing occurred after the P10 structural coiled-coil domain. Most importantly, processed P10 may be crucial for cell lysis to occur. We propose a mechanism in which the P10 perinuclear cage structure is cleaved by Cath after cell lysis was induced and the plasma membrane was damaged, which subsequently resulted nuclear disintegration.

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Chapter Four:

**The dual role for LEF-2 in
AcMNPV DNA replication and
very late gene expression**

4.1 Introduction

The early viral gene *lef-2* encodes for a protein essential for AcMNPV DNA replication (Kool *et al.*, 1994) by acting as a primase-associated factor able to bind ssDNA and potentially incorporating the viral primase LEF-1 into the replication complex (Introduction Figure 1.4; Mikhailov and Rohrmann, 2002).

Furthermore, it was suggested that LEF-2 plays a role in viral very late gene expression as either determined by transient expression assays (Passarelli and Miller, 1993; Srira and Gopinathan, 1998) or by random AcMNPV mutagenesis studies with BrdU (Merrington *et al.*, 1996). Mutagenesis of AcUW1.*lacZ* (*p10* coding region replaced by *lacZ*) identified a virus that was deficient in the activation of viral very late gene expression by screening for polyhedrin negative and colourless plaques after X-Gal staining. This virus was named very late deficient 1 (VLD1). Marker rescue experiments mapped the mutation responsible for the VLD1 phenotype to the AcMNPV *EcoR*-I fragment (7.3 kbp fragment, genome coordinates: 429-7760). Sequencing of the *EcoR*-I region of VLD1 revealed two point mutations inside this fragment within *bro* [position 1688; L144 phenylalanine (Y)] and *lef-2* (position 3620; D178N). However, only *lef-2* and not *bro* provided by co-transfection was able to rescue VLD1 very late gene expression as determined by marker rescue. Moreover, a virus (Ac*lef-2*³⁶²⁰; in this thesis referred to as AcLEF-2^{D178Npolh-}) generated with the very same point mutation as *lef-2* VLD1 produced BVs titres comparable to those from VLD1-infected cells. Their BV titre was approximately 10-fold lower than the titre of AcUW1.*lacZ*-infected cells at 48 h p.i. Viral DNA synthesis was not affected in either VLD1 or AcLEF-2^{D178Npolh-}-infected cells as indicated by dot blot analysis and by a *DpnI* sensitivity assay. It was therefore suggested that LEF-2^{D178N} was required for viral very late gene expression. However, the effect of AcLEF-2^{D178Npolh-} on *p10* and *polh* expression was not analysed (Merrington *et al.*, 1996).

In addition, the internal deletion of the Cys-rich domain in BmNPV *lef-2* by *SaI* digestion led to a loss of the trans-activation from the *polh* promoter as examined by the activity of the reporter gene *luc* (Sriram and Gopinathan, 1998).

The potential dual role of *lef-2* in viral replication and very late gene expression makes it a candidate gene required for the regulation of viral late to very late gene expression. Therefore, this chapter aims to investigate the function of LEF-2 in more detail by mutagenesis studies.

4.2 LEF-2 function prediction

To investigate the logical structure of *lef-2* function a gene ontology (GO) analysis was performed classifying the predicted gene function on the three hierarchical levels of

biological process, cellular component and molecular function (Fig. 4.1; Hamp *et al.*, 2013). With a 56% reliability, the LEF-2 biological processes includes: viral gene expression, transcription, nucleic acid processing and RNA biosynthesis. In addition, it was predicted that LEF-2 was part of the replisome complex by being part of the primosome (Fig. 4.1A) and having DNA primase activity (Fig. 4.1B). Moreover, a second molecular function of LEF-2 was assigned by binding to 4 iron- 4 sulphur (4Fe-4S) clusters (Fig. 4.1B). The primary role of 4Fe-4S involves the mediation of intracellular biological electron transport. However, they can also play a role in transcriptional or translational regulation of gene expression (reviewed by Johnson *et al.*, 2005). This further suggested a role of LEF-2 in both viral DNA replication and regulation of gene expression. Therefore, mutagenesis studies on AcMNPV *lef-2* were performed to investigate its potential dual function.

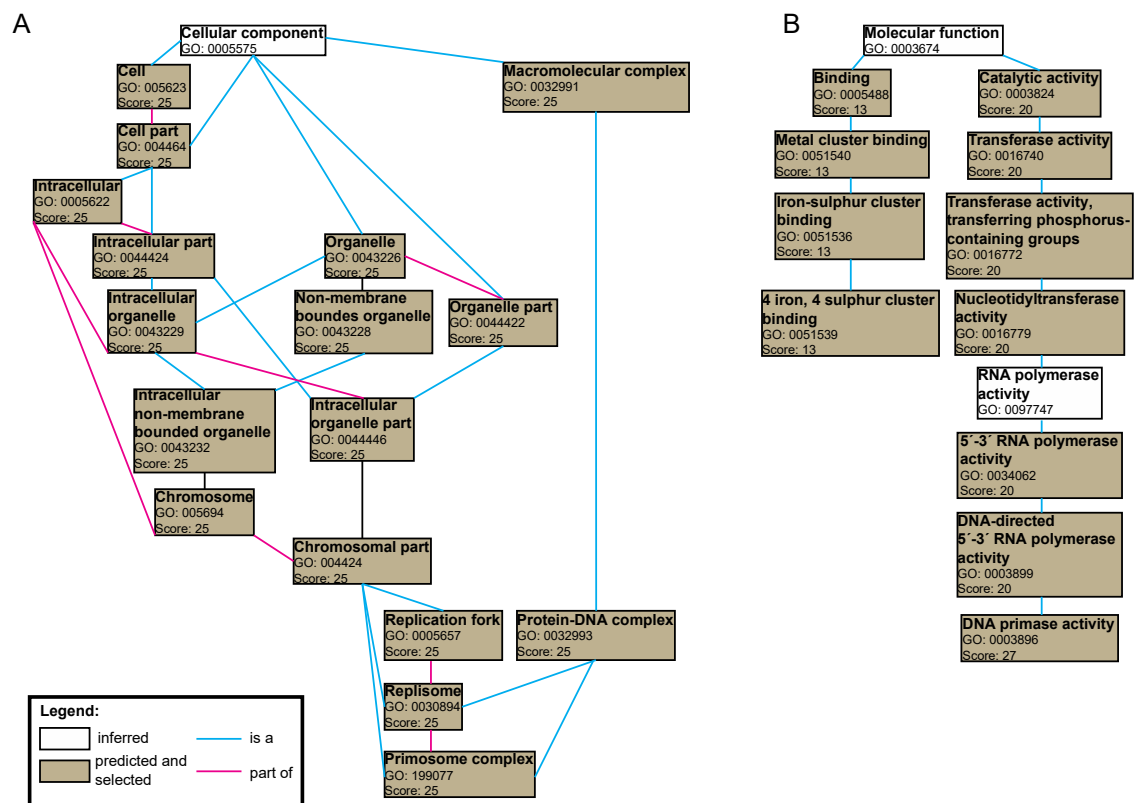


Figure 4.1 LEF-2 Gene Ontology (GO) analysis

GO analysis of LEF-2 for A) cellular component and B) molecular function ontology was performed using ProteinPredict (Hamp *et al.*, 2013). The GO term as well as the reliability (score) of the GO analysis is shown.

4.3 The role of AcMNPV *lef-2* in *p10* and *polh* expression

4.3.1 The effect of VLD1 on viral very late gene expression

It was shown previously that *lef-2* is essential for the activation of viral very late gene expression in *Sf21* as determined by marker rescue experiments with VLD1 (Merrington *et*

al., 1996). To confirm these results and to determine if very late gene expression is also deficient in *T. ni* Hi5 cells, VLD1-infected cell lysates were analysed on a protein gel (Fig. 4.2). Cells infected with AcMNPV accumulated both P10 and polyhedrin. The very late protein accumulation increased during later time points p.i. The VLD1 parental virus AcUW1.*lacZ* accumulated polyhedrin and β -Gal instead of P10 as *lacZ* was expressed under the *p10* promoter. However, VLD1-infected cells neither accumulated P10 nor β -Gal confirming that VLD1 abolished viral very late gene expression (Fig. 4.2; Merrington *et al.*, 1996).

To determine that VLD1 did not contain a mutation in the very late genes but was deficient in the activation of very late gene expression, a complementation assay was performed. During a complementation assay cells are co-infected with two viruses, whereby one virus provides the factors required for gene expression from the second (deficient) virus. The complementing virus used in this study was AcGFP, whose genome neither encoded for *lacZ* nor *polh* but expressed *gfp* under the *polh* promoter (Fig. 4.3A). Therefore, AcGFP-infected *T. ni* Hi5 cells neither accumulated polyhedrin nor β -Gal as determined by running the cell lysates on a SDS-PAGE. However, GFP (~24 kDa) accumulated instead of polyhedrin in AcGFP-infected cells (Fig. 4.3B). Importantly, this virus provided the factors necessary for the activation of AcMNPV very late gene expression. Co-infection of AcGFP with VLD1 resulted in the accumulation of both β -Gal and polyhedrin on a protein gel further indicating that VLD1 was deficient in the activation of viral very late gene expression (Fig. 4.3B).

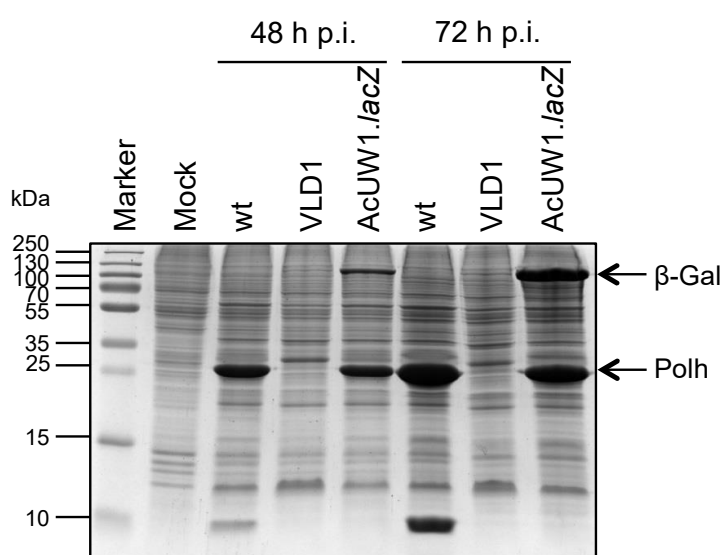


Figure 4.2 VLD1 infection of *T. ni* Hi5 cells

T. ni Hi5 cells were infected with wild-type (wt) AcMNPV, VLD1 or, as a positive control for VLD1 with its parental virus AcUW1.*lacZ* at MOI 3 to analyse their effect on viral very late gene expression. As a negative control cells were mock-infected. Cells were harvested either at 48 h p.i. or 72 h p.i. Protein samples were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight β -galactosidase (β -Gal) and polyhedrin (Polh).

It was noted that P10 (Fig. 4.3B) appeared not to accumulate as strongly at 72 h p.i. as at the same time point p.i. shown before (Fig. 4.2). The reason for this may be that the protein gel was not fixed properly and P10 diffused out of the gel.

4.3.2 The role of AcLEF-2^{D178N} on very late gene expression

Mutagenesis with BrdU does not necessarily lead to a single point mutation in genomes (Hopkins and Goodman, 1980). To validate that the mutation LEF-2^{D178N} identified in VLD1 was required for very late gene expression, the same amino acid change was introduced into the AcMNPV genome into a *polh* negative backbone (Merrington *et al.*, 1996). As the effect of the LEF-2^{D178N} on very late gene expression was not analysed previously its effect on *p10* expression was examined in this study. Surprisingly, infection of both *Sf9* and *T. ni* Hi5 cells with AcLEF-2^{D178N}_{polh-} resulted in P10 accumulation from 48 h p.i. until 96 h p.i. at levels comparable to wt-infected cells (Fig. 4.4).

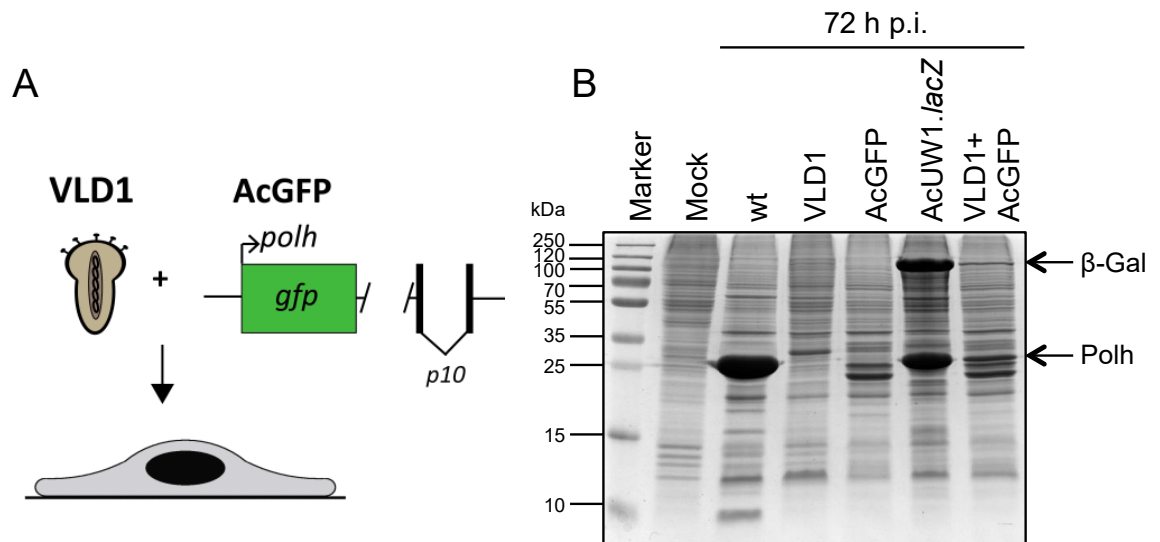


Figure 4.3 VLD1 is deficient in the activation of viral very late gene expression

A, B) *T. ni* Hi5 cells were co-infected with VLD1 and its complementing virus AcGFP at MOI 3 to confirm the deficiency of VLD1 in very late gene expression. B) Cells infected with either wild-type (wt) AcMNPV, VLD1, AcUW1.*lacZ* or mock-infected cells served as controls. Cells were harvested 72 h p.i. and proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight β -galactosidase (β -Gal) and polyhedrin (Polh).

To further examine the effect of LEF-2^{D178N} on *polh* expression, *polh* was reinserted into AcLEF-2^{D178N}_{polh-} DNA by co-transfection with the *polh* fragment into *Sf9* cells followed by one round of plaque purification and amplification of polyhedra-positive plaques to generate a P1 stock. Successful generation of AcLEF-2^{D178N} DNA was verified by PCR (data not shown).

Both *Sf9* and *T. ni* Hi5 cells infected with AcLEF-2^{D178N} accumulated the viral very late proteins polyhedrin and P10 from 48 h p.i.-96 h p.i. to a similar amount as in wt-infected

(Fig. 4.5). This indicated that AcMNPV the mutation LEF-2^{D178N} did not affect viral very late gene expression.

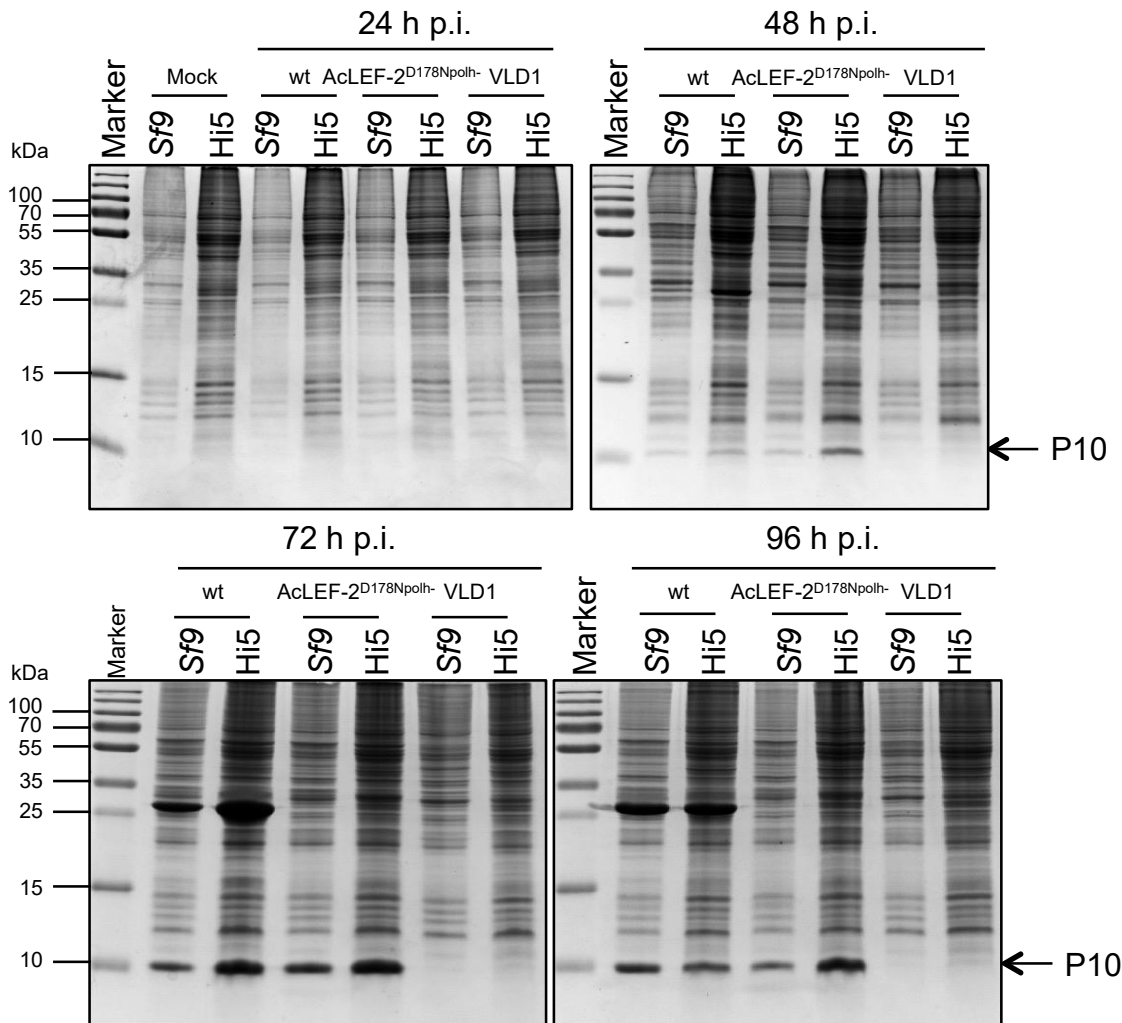


Figure 4.4 Time course of AcLEF-2^{D178Npolh}- and VLD1-infected cells

Sf9 and *T. ni* Hi5 (Hi5) cells were infected with AcLEF-2^{D178Npolh}- or VLD1 at MOI 3 to determine their effects on viral very late gene expression. As controls cells were infected with wild-type (wt) AcMNPV or were mock-infected. Cells were harvested every 24 h over a period from 24-96 h p.i. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrow highlights accumulated P10.

4.3.3 The effects of AcLEF-2^{A154T} on *p10* and *polh* expression

Surprisingly, as LEF-2^{D178N} did not affect AcMNPV very late gene expression, it was decided to resequence the *lef-2* orf from VLD1. Reanalysis of VLD1 *lef-2* revealed a second so far undetected mutation at AcMNPV genome position 3548 (from guanine to adenine) that resulted in an amino acid change from alanine to threonine in LEF-2 (LEF-2^{A154T}).

Therefore, the hypothesis was tested that the LEF-2^{A154T} mutation was responsible for the phenotype observed after cells were infected with VLD1. Hence, the very same point

mutation was introduced into the AcMNPV genome by crossover PCR using Ac Δ lef-2 bacmid DNA (kindly provided by A. Chambers) as a backbone (Fig. 4.6). The successful modification of *lef-2* in pBS.SK+CB4 was confirmed by Sanger sequencing (Source BioScience, data not shown).

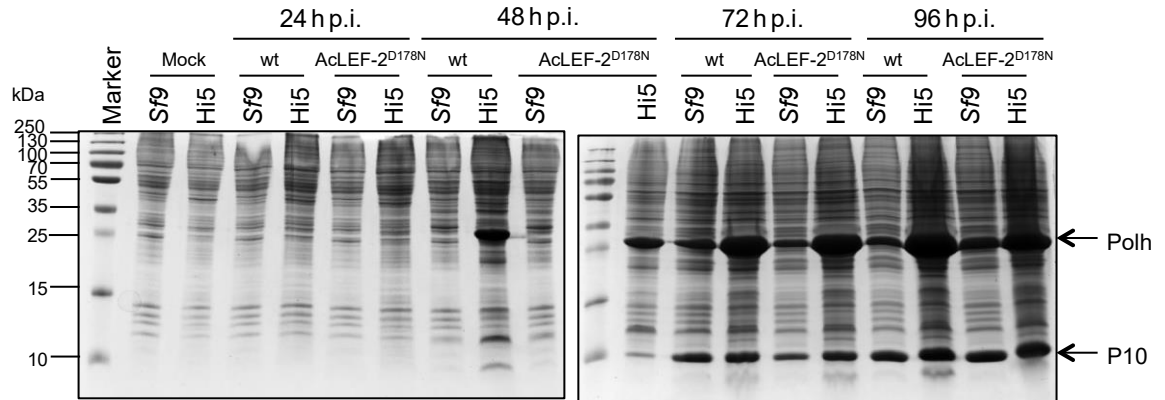


Figure 4.5 Time course of AcLEF-2^{D178N}-infected cells

Sf9 and *T. ni* Hi5 cells were infected with AcLEF-2^{D178N} at MOI 3 to determine its effects on viral very late gene expression. As controls, cells were infected with wild-type (wt) AcMNPV or they were mock-infected. Cells were harvested every 24 h over a period of 96 h p.i. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight polyhedrin (Polh) and P10.

The Ac Δ lef-2 bacmid DNA (*polh* negative backbone) contained a unique *SrfI* restriction site. As *lef-2* was determined to be essential for virus replication (Kool *et al.*, 1994) and linearised baculovirus DNA greatly improved the yield of recombinant virus and additionally reduced background contamination by the parental virus (Kitts *et al.*, 1990), linearised Ac Δ lef-2 DNA was co-transfected with pBS.SK+CB4 in *Sf21* cells to generate the recombinant AcLEF-2^{A154T}. Surprisingly, even 7 d p.t. no cytopathic effects (cpe) could be observed in co-transfected cells (data not shown). However, when linearised Ac Δ lef-2 was rescued by co-transfection with a plasmid (pAcEI-I) encoding for the AcMNPV *EcoR*-I fragment including wt *lef-2*, polyhedrin accumulated inside the nucleus of co-transfected cells and progeny virus was generated. In addition, when cells were transfected with either AcLEF-2^{D178N,polh-} or VLD1 (LEF-2^{A154T,D178N}) DNA, cpe were observed and BVs were produced (data not shown).

To detect any underlying low level of virus replication, attempts were made to amplify AcLEF-2^{A154T} in *Sf21* monolayer cells by passaging any recombinant virus from the initial co-transfection twice (P2 virus stock) but no cpe could be detected even after 9 d p.i. (data not shown). To verify that no virus was present inside the AcLEF-2^{A154T} P2 stock, virus DNA was extracted at 9 d p.i. and screened for *lef-2* and *gp64* DNA by PCR. Although virus DNA was present after DNA extraction from wt-infected cells, neither *lef-2* nor *gp64* DNA could be amplified from the DNA extract of the P2 stock of AcLEF-2^{A154T}.

infected cells (data not shown). Collectively, these results indicate that LEF-2^{A154T} might be lethal for AcMNPV protein function.

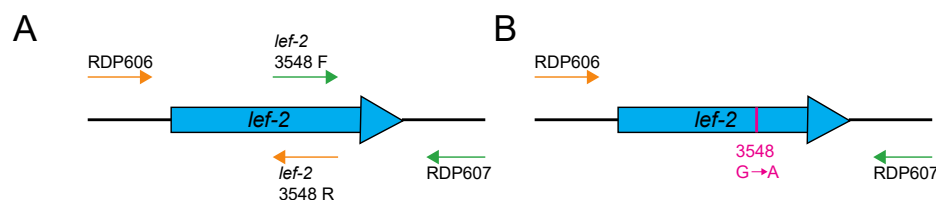


Figure 4.6 Generation of AcLEF-2^{A154T}

The point mutation at AcMNPV genome position 3548 inside LEF-2 was generated by crossover PCR. A) During the first round of PCR the point mutation G→A at *lef-2* position 3548 leading to a A154T amino acid change in the translated protein product was inserted. Wild-type DNA was used as a template and two independent PCR reactions were performed by using the primer pairs: RDP606/*lef-2* 3548 R (orange) and *lef-2* 3548 F/RDP607 (green). The primers *lef-2* 3548 F and R overlap and both contain the desired mutation in their sequence. B) During the second round of PCR *lef-2* 3548 was amplified by combining the PCR products from the first round using the primer pair RDP606 and RDP607. The PCR product was inserted into the *Hinc*II digestion site of pBS.SK+ thereby generating the transfer vector pBS.SK+CB4. The DNA sequence of the transfer vector was verified by Sanger sequencing (Source BioScience, data not shown). AcLEF-2^{A154T} was generated by the co-transfection of linearised AcΔ*lef-2* DNA (kindly provided by A. Chambers) with the transfer vector pBS.SK+CB4.

4.3.4 LEF-2 conservation and predicted protein structure

So far, it has been determined that AcMNPV LEF-2^{D178N} was not required for viral very late gene expression, while LEF-2^{A154T} seemed to be non-permissive for protein function. At the same time a combination of both mutations inside LEF-2 was permissive for virus replication but not very late gene expression. Therefore, to gain a more detailed picture of the LEF-2 protein, the conservation of baculovirus LEF-2 amino acids (Table 4.1) was investigated by T-coffee alignment (supplementary CD Fig. S 4.1; Notredame *et al.*, 2000).

The alignment of all available LEF-2 amino acid sequences revealed that alanine 154 is highly conserved in 91% of all analysed baculoviruses, whereby the LEF-2 aspartic acid 178 is not conserved across baculoviruses (only 23% conservation in all baculoviruses and 35% in alphabaculoviruses; Table 4.2) suggesting a crucial role for LEF-2^{A154} in protein function.

Table 4.1 Available baculovirus LEF-2 amino acid sequences*

Name	LEF-2 length (aa)	Accession number
Alphabaculovirus		
Autographa californica multiple nucleopolyhedrovirus	210	AAC17846.1
Adoxophyes honmai nucleopolyhedrovirus	211	NP_818747.1
Agrotis ipsilon multiple nucleopolyhedrovirus	215	ACI28719.1
Agrotis segetum nucleopolyhedrovirus A	210	YP_529683.1
Agrotis segetum nucleopolyhedrovirus B	214	YP_009112575.1
Antheraea pernyi nucleopolyhedrovirus	246	AAU89800.1

Table 4.1 Continued: Available baculovirus LEF-2 amino acid sequences

<i>Philosamia cynthia ricini</i> nucleopolyhedrovirus virus	246	AFY62942.1
<i>Anticarsia gemmatilis</i> multiple nucleopolyhedrovirus	204	ALR72482.1
<i>Anagrapha falcifera</i> MNPV	210	AAB53355.1
<i>Plutella xylostella</i> multiple nucleopolyhedrovirus	210	ABE68391.1
<i>Rachiplusia ou</i> MNPV	210	AAC33756.1
<i>Bombyx mori</i> nucleopolyhedrovirus	210	NP_047556.1
<i>Bombyx mandarina</i> nucleopolyhedrovirus	210	ACQ57331.1
<i>Buzura suppressaria</i> nucleopolyhedrovirus	210	AAC77814.1
<i>Choristoneura fumiferana</i> DEF multiple nucleopolyhedrovirus	203	NP_932612.1
<i>Choristoneura fumiferana</i> multiple nucleopolyhedrovirus	202	NP_848315.1
<i>Choristoneura occidentalis</i> alphabaculovirus	202	AGR57036.1
<i>Choristoneura murinana</i> nucleopolyhedrovirus	202	YP_008992237.1
<i>Chrysodeixis chalcites</i> nucleopolyhedrovirus	226	AGE61845.1
<i>Chrysodeixis chalcites</i> SNPV TF1-A	226	AGC36350.1
<i>Chrysodeixis includens</i> nucleopolyhedrovirus	224	AOL57273.1
<i>Pseudoplusia includens</i> SNPV IE	223	YP_009117042.1
<i>Clanis bilineata</i> nucleopolyhedrovirus	220	YP_717647.1
<i>Dasychira pudibunda</i> nucleopolyhedrovirus	204	AKR14232.1
<i>Ectropis obliqua</i> nucleopolyhedrovirus	211	AGS47948.1
<i>Epiphyas postvittana</i> nucleopolyhedrovirus	203	NP_203173.1
<i>Euproctis pseudoconspersa</i> nucleopolyhedrovirus	215	YP_002854728.1
<i>Helicoverpa armigera</i> NPV NNg1	226	BAG74688.1
<i>Helicoverpa armigera</i> NPV strain Australia	71	AEN04040.1
<i>Helicoverpa armigera</i> nucleopolyhedrovirus G4	241	AAG53860.1
<i>Helicoverpa armigera</i> SNPV	238	AIG63299.1
<i>Helicoverpa</i> SNPV AC53	238	AMN16382.1
<i>Helicoverpa zea</i> single nucleopolyhedrovirus	238	AAG17379.1
<i>Hyphantria cunea</i> nucleopolyhedrovirus	209	YP_473335.1
<i>Leucania separata</i> nucleopolyhedrovirus	254	YP_758434.1
<i>Lymantria dispar</i> multiple nucleopolyhedrovirus	216	AMO27992.1
<i>Mamestra brassicae</i> multiple nucleopolyhedrovirus	215	YP_009011078.1
<i>Mamestra configurata</i> nucleopolyhedrovirus A	211	NP_613097.1
<i>Mamestra configurata</i> nucleopolyhedrovirus B	215	AAM95000.1
<i>Helicoverpa armigera</i> multiple nucleopolyhedrovirus	215	ACH88535.1
<i>Maruca vitrata</i> nucleopolyhedrovirus	210	YP_950856.1
<i>Orgyia pseudotsugata</i> multiple nucleopolyhedrovirus	204	NP_046162.1
<i>Spodoptera exigua</i> multiple nucleopolyhedrovirus	209	CDG73193.1
<i>Spodoptera frugiperda</i> multiple nucleopolyhedrovirus	213	AFH58983.1
<i>Spodoptera littoralis</i> nucleopolyhedrovirus	258	AGE89965.1
<i>Spodoptera litura</i> nucleopolyhedrovirus	210	NP_258382.1
<i>Thysanoplusia orichalcea</i> nucleopolyhedrovirus	211	YP_007250418.1
<i>Trichoplusia ni</i> single nucleopolyhedrovirus	225	YP_309017.1
<i>Adoxophyes orana</i> nucleopolyhedrovirus	240	YP_002300613.1
<i>Amsacta albistriga</i> nucleopolyhedrovirus	206	AAD26699.1
<i>Anticarsia gemmatilis</i> nucleopolyhedrovirus	204	CAC03566.1
<i>Apocheima cinerarium</i> nucleopolyhedrovirus	234	YP_006607856.1
<i>Condylorrhiza vestigialis</i> MNPV	204	YP_009118620.1
<i>Dendrolimus kikuchii</i> nucleopolyhedrovirus	228	AFS52022.1
<i>Iragoides fasciata</i> nucleopolyhedrovirus	215	ACJ04626.1
<i>Lambdina fiscellaria</i> nucleopolyhedrovirus	250	YP_009133233.1
<i>Lonomia obliqua</i> multiple nucleopolyhedrovirus	220	AKN81016.1
<i>Lymantria xylinia</i> MNPV	210	YP_003517858.1
<i>Mamestra configurata</i> nucleopolyhedrovirus A	211	NP_613097.1
<i>Mamestra configurata</i> nucleopolyhedrovirus B	215	NP_689188.1
<i>Orgyia ericae</i> nucleopolyhedrovirus	215	CAF32809.1
<i>Orgyia leucostigma</i> NPV	210	YP_001651024.1

Table 4.1 Continued: Available baculovirus LEF-2 amino acid sequences

Orgyia pseudotsugata single capsid nucleopolyhedrovirus	204	BAA08770.1
Peridroma alphabaculovirus	209	YP_009049837.1
Perina nuda nucleopolyhedrovirus	204	AAD48439.1
Spodoptera litura nucleopolyhedrovirus II	211	YP_002332712.1
Sucra jujuba nucleopolyhedrovirus	229	YP_009186801.1
Hemileuca sp. nucleopolyhedrovirus	221	YP_008378330.1
Betabaculoviruses		
Adoxophyes orana granulovirus	170	NP_872486.1
Agrotis segetum granulovirus	181	AKN63313.1
Closteria anachoreta granulovirus	176	YP_004376237.1
Closteria anastomosis granulovirus Henan	176	YP_008719978.1
Closteria anastomosis granulovirus B	184	AKS25372.1
Cryptophlebia leucotreta granulovirus	168	NP_891885.1
Cydia pomonella granulovirus	171	NP_148825.1
Epinotia aporema granulovirus	127	YP_006908549.1
Helicoverpa armigera granulovirus	189	YP_001649015.1
Phthorimaea operculella granulovirus	180	NP_663202.1
Plodia interpunctella granulovirus	172	YP_009330166.1
Plutella xylostella granulovirus	270	AMQ35878.1
Pseudaletia unipuncta granulovirus	194	YP_003422372.1
Trichoplusia ni granulovirus LBIV-12	194	AOW41369.1
Spodoptera litura granulovirus	174	YP_001256983.1
Trichoplusia ni granulovirus	194	AOW41369.1
Xestia c-nigrum granulovirus	189	NP_059183.1
Choristoneura occidentalis granulovirus	188	YP_654450.1
Cnaphalocrocis medinalis granulovirus	63	ALN41958.1
Diatraea saccharalis granulovirus	172	YP_009182234.1
Erinnyis ello granulovirus	175	YP_009091873.1
Mythimna unipuncta granulovirus	192	AQQ80303.1
Pieris rapae granulovirus	172	ADO85459.1
Spodoptera frugiperda granulovirus	198	YP_009121815.1
Mocis sp. granulovirus	188	YP_009249870.1
Gammabaculovirus		
Neodiprion lecontei nucleopolyhedrovirus	195	AAQ99100.1
Neodiprion sertifer nucleopolyhedrovirus	200	YP_025164.1
Neodiprion abietis NPV	195	YP_667905.1
Dealtabaculovirus		
Culex nigripalpus nucleopolyhedrovirus	225	NP_203329.1
Unclassified Baculovirus		
Perigonia lusca single nucleopolyhedrovirus	209	YP_009165717.1
Urbanus proteus nucleopolyhedrovirus	217	AKR17323.1

*List adapted from NCBI Entrez Genome (May 2017); Abbreviation: aa: amino acid

During the analysis of the conserved LEF-2 amino acids a few more that were highly conserved were identified inside LEF-2 (>90% conservation). These amino acids include five cysteine residues at the LEF-2 C-terminus at positions 84, 143, 146, 150 and 165 and in addition tyrosine 24, 159, phenylalanine 43, glycine 47, leucine 57, arginine 130 and lysine 164 (Table 4.2).

To further examine how LEF-2 A154 and D178 are organised within the protein an *in silico* analysis of the structure using the bioinformatics program Phyr² was performed (Fig. 4.7; Kelly *et al.*, 2015). With a very high confidence A154 was determined to be part of an α -helix, while D178 was not part of any secondary protein structure (pink boxes, Fig. 4.7A).

The modelling was performed with references to known protein structures, which were used as backbones. Unfortunately, the program is unable to accurately determine the structural effect of a point mutation as the mutation will have no alterations on the backbone itself. It would only change the side chain of an amino acid. However, the program could predict the likelihood of a phenotypic effect of the possible point mutations of an amino acid (Kelly *et al.*, 2015). While the LEF-2^{A154T} was predicted to very likely affect protein function, the mutation LEF-2^{D178N} was not likely to affect function (data not shown). Overall, it was hypothesised that AcMNPV LEF-2^{D178N} and LEF-2^{D178N,A154T} affected protein tertiary structure in a way that was acceptable for protein function, while LEF-2^{A154T} did not.

Table 4.2 The most highly conserved LEF-2 amino acids

LEF-2 amino acid residue ¹	Conservation across all baculoviruses ² (%)
Mutations in VLD1	
Ala154	91% (96% in alphabaculoviruses)
Asp178	23% (35% in alphabaculoviruses)
Cysteine residues (Allen, 2006)	
Cys84	97%
Cys88	28% (38% in alphabaculoviruses)
Cys146	96%
Other highly conserved Cysteine residues (>95% conservation)	
Cys143	99%
Cys150	99%
Cys165	98%
Other highly conserved amino acids (>90% conservation)	
Tyr24	93%
Phe43	94%
Gly47	92%
Leu57	94%
Arg130	99%
Tyr159	98%
Lys164	98%

¹with respect to AcMNPV LEF-2 sequence; ²across all available (99) LEF-2 sequences in May 2017

4.3.5 The effect of AcLEF-2^{A154T,D178N} on very late gene expression

To investigate if the combination of VLD1 LEF-2^{A154T,D178N} could reproduce the reduction in viral very late gene expression as seen in VLD1-infected cells both point mutations were inserted into the AcMNPV genome by generating both a *polh*-negative and *polh*-positive virus. It was decided to analyse the *lef-2* mutations in both *polh* negative and positive contexts as VLD1 encoded for *polh* but also to control for the lethal effect observed in AcLEF-2^{A154T}, which had been generated in a *polh* negative backbone. Therefore, either the *lef-2* orf or the *lef-2*, *orf603* and *polh* fragment from VLD1 were PCR amplified and inserted into the *Bam*HI restriction site of pBS.SK+ producing pBS.SK+CB3 and pBS.SK+CB4, respectively. For virus generation, *Srf*I digested bacmid AcΔ*lef-2* DNA was rescued with the appropriate transfer vector and viruses were subsequently amplified without any plaque purification. The reason for the direct amplification was that *lef-2* is

essential for viral DNA replication (Kool *et al.*, 1994), which was not encoded by Ac Δ lef-2, and furthermore, linearised baculovirus DNA is about 15-150-fold less infectious than circular virus DNA (Kitts *et al.*, 1990). The DNA sequence of the transfer vectors and the *lef-2* orfs of the newly generated viruses were verified by Sanger sequencing (Source BioScience, data not shown). However, to control for any remaining parental background contaminations and because it was noted that Ac Δ lef-2 was able to produce tiny plaques during titration assays (R. D. Possee, personal communication), linearised Ac Δ lef-2 was also rescued by co-transfection with pAcEI-I encoding for wt *lef-2*. The resulting virus was named Ac Δ lef-2^{rescue}.

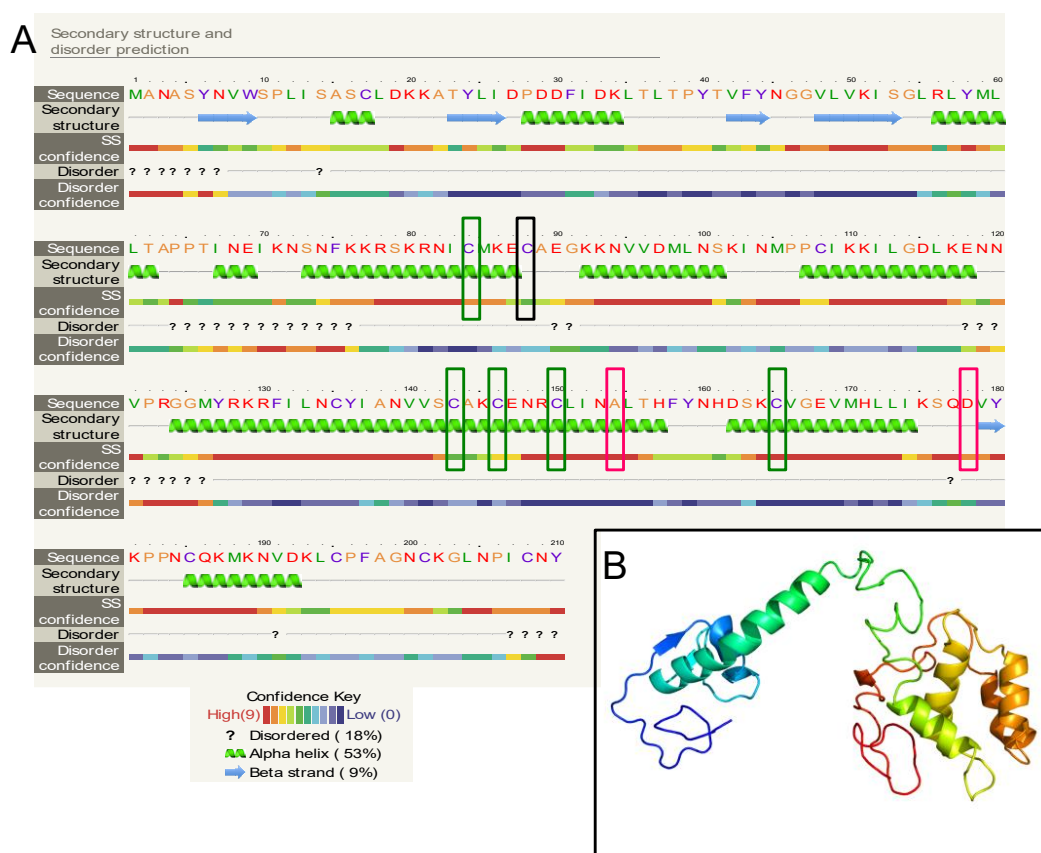


Figure 4.7 AcMNPV LEF-2 secondary and tertiary *in silico* structure

AcMNPV LEF-2 predicted A) secondary and B) tertiary structure using the bioinformatics program Phyr² (Kelly *et al.*, 2015). A) The five most highly conserved cysteine residues (C84, C143, C146; C150, C165) are boxed in green, the *lef-2* amino acids mutated in VLD1 in pink (A154, D178) and the non-conserved cysteine residue 88 in black. B) The 3D structure of 84% (176aa) of LEF-2 was modelled with >90% accuracy (Coordinates in Å: X: 61.433, Y: 47.715, Z: 65.566); Image coloured by rainbow N→C. Lowest confidence for the tertiary structure is at the very N- and C-terminus and in between α -helices and β -strands. Highest confidences are α -helices and β -strands. The protein is predicted to persist of 53% α -helices, 9% β -strands and 18% disordered structure. The main templates for backbone generation were the crystal structures of 1) fe-s domain of yeast DNA primase, 2) large subunit of human DNA primase, and 3) the human polymerase alpha-primase p58 iron-sulphur 2 cluster domain

To confirm that Ac Δ lef-2 produced infectious progeny, the virus was amplified in monolayer culture and *Sf21* cells were infected with 500 μ l of the P0 virus inoculum. At

96 h p.i. virus DNA was extracted from BV in the medium and screened for *gp64* and *lef-2* DNA by PCR (Fig. 4.8). Gel electrophoresis of the PCR products revealed bands specific for *gp64* after DNA extraction from the cell culture medium of both wt and *AcΔlef-2*-infected cells. Furthermore, PCR amplification using *lef-2* specific primers amplified an 819 bp DNA fragment after cells were infected with wt, while an 1150 bp DNA fragment was amplified after *AcΔlef-2* infection. The size of this DNA fragment corresponds to the neomycin-expressing cassette, which replaced the *lef-2* coding sequence in *AcΔlef-2* (compare Introduction Fig. 1.10). This suggested that progeny viruses were produced even when the *lef-2* orf was deleted from the AcMNPV genome.

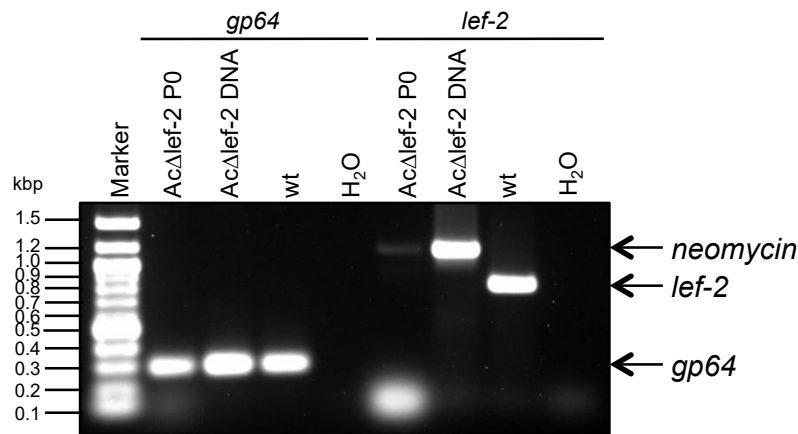


Figure 4.8 *Gp64* and *lef-2* DNA amplification of *AcΔlef-2* P0

Virus extracted DNA from either *AcΔlef-2* P0 or as control from *AcΔlef-2* bacmid DNA, wild-type (wt) AcMNPV or the H₂O-negative control were PCR amplified using a primer pair (qPCR *gp64* F2/R4) specific for *gp64* or *lef-2* (RDP 606 and RDP 607). The *gp64* primer pair amplified a 292 bp DNA fragment, while the *lef-2* primer pair amplified an 816 bp fragment from wt DNA or an 1150 bp fragment from *AcΔlef-2* extracted DNA, in which the *lef-2* orf was exchanged for neomycin.

To examine how AcMNPV LEF-2^{A154T,D178N} affected very late gene expression, both *Sf9* and *T. ni* Hi5 cells were infected with either AcLEF-2^{A154T,D178N,polh-} or AcLEF-2^{A154T,D178N}, which both contain the double mutation. Infected AcLEF-2^{A154T,D178N,polh-} or AcLEF-2^{A154T,D178N} cells accumulated P10 to a level comparable to *AcΔlef-2*^{rescue}-infected cells from 48-96 h p.i. (Fig. 4.9). In addition, polyhedrin accumulated in AcLEF-2^{A154T,D178N}-infected *Sf9* and *T. ni* Hi5 cells to an amount comparable to *AcΔlef-2*^{rescue}-infected cells at each analysed time point p.i. suggesting that the mutation LEF-2^{A154T,D178N} does not affect very late gene expression. However, P10 and polyhedrin accumulation was lowered in both cell lines analysed after *AcΔlef-2*^{rescue} infection when compared to wt-infected cells (Fig. 4.9). This indicated either a parental background contamination inside the *AcΔlef-2*^{rescue} stock resulting from undigested or religated virus DNA or another mutation inside *AcΔlef-2*^{rescue}, which affected very late gene expression. Therefore, any further viruses generated to analyse the function of LEF-2 using a linearised *AcΔlef-2* as a backbone were plaque purified to decrease the chance of parental virus contaminations.

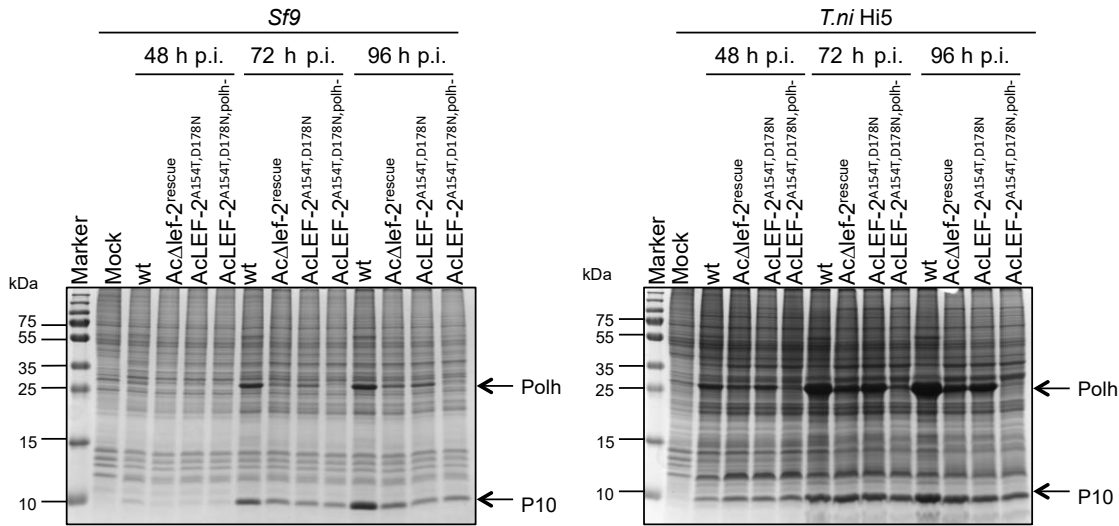


Figure 4.9 Time course of AcLEF-2^{A154T,D178N,polh-} and AcLEF-2^{A154T,D178N}-infected cells

Sf9 and *T. ni* Hi5 cells were infected with AcLEF-2^{A154T,D178N,polh-} or AcLEF-2^{A154T,D178N} at MOI 3 to determine their effects on viral very late gene expression. As controls cells were infected with wild-type (wt) AcMNPV, AcΔlef-2^{rescue} or were mock-infected. Cells were harvested every 24 h over a period from 48-96 h p.i. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight polyhedrin (Polh) and P10.

4.3.6 VLD1 rescue experiments

Surprisingly, AcMNPV LEF-2^{A154T,D178N} did not affect viral very late gene expression. Hence, it was determined if the phenotype caused by VLD1 could be rescued by the viral genes encoded by the AcMNPV *EcoR*-I region (Merrington *et al.*, 1996). The 7.3 kbp *EcoR*-I fragment encodes for the following viral genes: *ptp*, *bro*, *ctx*, *orf4*, *orf5*, *lef-2*, *orf603*, *polh*, *orf1629* and *pk-1*. Marker rescue experiments were performed by the co-transfection of VLD1 DNA with pAcEI-I (encoding the AcMNPV *EcoR*-I region) or as a positive control VLD1 DNA was co-transfected with AcΔpolh DNA in *Sf21* cells. In contrast to previous published work (Merrington *et al.*, 1996) neither polyhedrin nor blue staining of the culture medium from co-transfected cells after X-Gal staining was observed at 3 d p.t. However, at 7 d p.t. the medium of co-transfected cells turned blue after X-Gal staining indicating *lacZ* expression from the *p10* promoter although cells did not contain any polyhedra as determined by light microscopy (data not shown). However, BV (from the cell culture medium) was harvested, plaque purified and blue plaques were amplified after X-Gal staining generating VLD1^{AcΔpolh} or VLD1^{EcoR-I}, respectively.

Both *Sf9* and *T. ni* Hi5 cells infected with VLD1^{AcΔpolh} accumulated P10 and polyhedrin to a similar level as wt-infected cells from 48 h p.i.-96 h p.i. (Fig. 4.10). This indicated that AcΔpolh could fully rescue VLD1 very late gene expression although a small accumulation of β-Gal was detectable after running VLD1^{AcΔpolh}-infected *T. ni* Hi5 cell lysates on a SDS-PAGE. The reason may be that the VLD1^{AcΔpolh} still contained a small portion of parental VLD1 as a background contamination as only one round of plaque purification was

performed. In addition, when either *Sf9* or *T. ni* Hi5 cells were infected with VLD1^{EcoR-I} accumulation of both polyhedrin and β -Gal occurred to a similar amount as in cells infected with AcUW1.*lacZ*, the VLD1 parental virus. Furthermore, β -Gal accumulation in AcUW1.*lacZ*-infected cells occurred at similar levels to another *lacZ*-expressing virus, AcRP23.*lacZ*, which was included as another control for β -Gal accumulation (Fig. 4.10). This suggested that the mutation responsible for the deficiency in VLD1 very late gene expression is present in the AcMNPV *EcoR-I* region.

As very late gene expression was fully rescued by *EcoR-I* and furthermore, the point mutations LEF-2^{A154T,D178N} (as found in VLD1) had no effect on either *p10* or *polh* expression, the VLD1 *EcoR-I* fragment was resequenced (Table 4.3). Reinvestigation of the sequence confirmed the point mutations inside the *lef-2* and *bro* orfs. The point mutation BRO^{L144Y} had previously been shown to be nonessential for very late gene expression as determined by marker rescue experiments of VLD1 with wt *bro* (Merrington *et al.*, 1996). Surprisingly, more mutations were found in the *pk-1* orf of VLD1 (Table 4.3). At AcMNPV genome position 6933 the nucleotide cytosine was exchanged by a thymine resulting in an alanine to valine mutation (PK-1^{A6V}). Moreover, a cytosine nucleotide was inserted after position 7465 and at the same time a thymine at genome position 7481 was deleted resulting in a series of amino acid changes in PK-1: T184 histidine (H), T185N, methionine (M) 186Y, H187A, V188 arginine (R). While PK-1^{A6V} sequencing results were verified by Sanger sequencing using both a forward and reverse primer, the C insertion and T deletion could only be verified by a single primer. Unfortunately, the 3' region of *pk-1* was not accessible for Sanger sequencing even when a different primer pair was used (Source Bioscience, personal communication). However, the sequencing results in combination with the time course experiment of AcLEF-2^{VLD-1} suggested that *pk-1* may be required or at least contributed to the deficiency of viral very late gene expression in VLD1.

It was shown previously that wt *lef-2* was able to rescue VLD1 very late gene expression as investigated by transient expression assays and marker rescue experiments (Merrington *et al.*, 1996). In the light of the newly found mutations inside VLD1 *pk-1*, it was reinvestigated if *lef-2* is required for very late gene expression. Therefore, the marker rescue experiments with VLD1 DNA and wt *lef-2* PCR DNA were repeated. However, this time it was attempted to isolate and amplify the newly generated virus (VLD1^{LEF-2}). Although the medium turned blue after X-Gal staining of co-transfected *Sf21* cells with VLD1 and wt *lef-2* DNA, no polyhedra were observed at 9 d p.t. by light microscopy (data not shown). Nevertheless, the medium of co-transfected cells was harvested, titrated and blue plaques were amplified after X-Gal staining. However, no BV could be amplified neither in *Sf9* shake nor in *Sf21* monolayer culture.

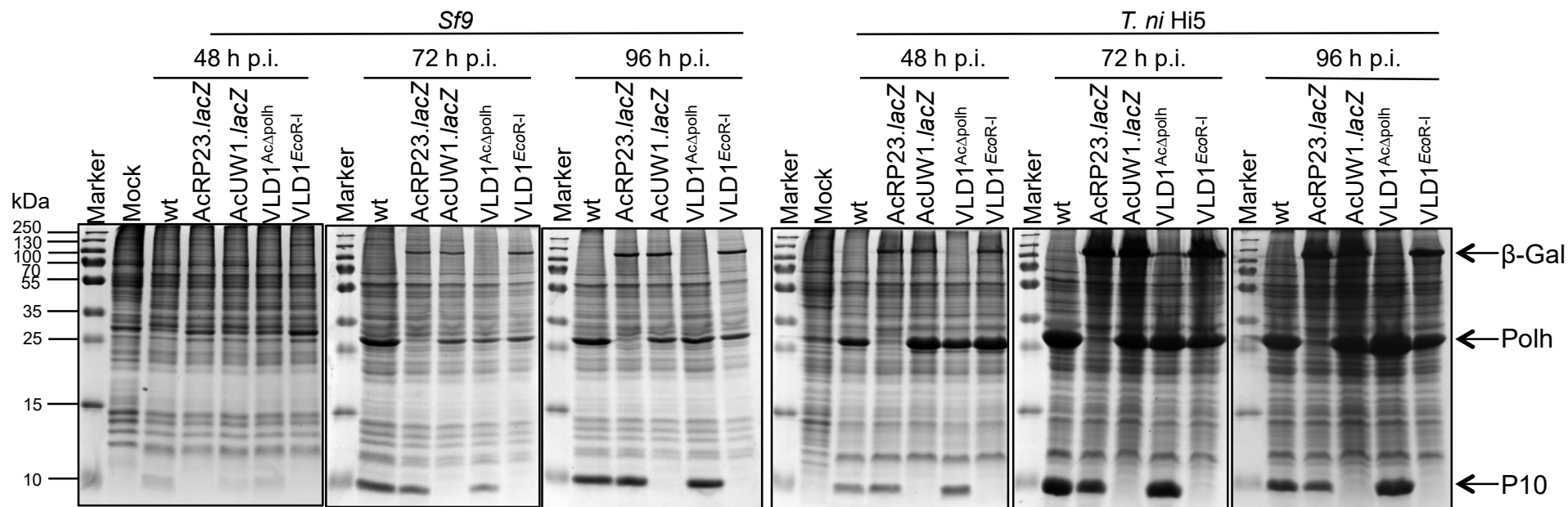


Figure 4.10 Time course of VLD1 rescue viruses

Sf9 and *T. ni* Hi5 cells were infected with VLD1^{AcΔpolh} or VLD1^{EcoR-I} at MOI 3 to determine their effects on viral very late gene expression. As controls cells were infected with wild-type (wt) AcMNPV, AcUW1.lacZ, AcRP23.lacZ or were mock-infected. Cells were harvested every 24 h over a period from 48-96 h p.i. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight β-galactosidase (β-Gal), polyhedrin (Polh) and P10.

4.3.7 The role of the conserved LEF-2 cysteine residues on viral very late gene expression

During the alignment of all available LEF-2 baculovirus sequences it was noted that five cysteine residues (C84, C143, C146, C150, C165) at the AcMNPV LEF-2 C-terminus were highly conserved (>95% conservation; Table 4.2; supplementary CD Fig. S 4.1). All five cysteine residues were predicted to be part of α -helices within protein secondary structures (green boxes, Fig. 4.7). Moreover, point mutations of any of these highly conserved cysteine residues were predicted to most likely have an effect on protein function (data not shown; Kelly *et al.*, 2015). As cysteine residues are often observed in functionally important sites of the protein (reviewed by Marino and Gladyshev, 2012), their importance on viral very late gene expression was investigated by mutagenesis studies.

Table 4.3 Sanger sequencing results of the VLD1 *EcoR*-I region

Gene	Base pair (AcMNPV genome position)	Amino acid
<i>bro</i> *	1688 C→T	Leu144Phe
<i>lef-2</i>	3548 G→A	Ala154Thr
<i>lef-2</i>	3620 G→A	Asp178Asn
<i>pk-1</i>	6933 C→T	Ala6Val
<i>pk-1</i> **	C insertion after 7465; T deletion at 7481	Thr184His, Thr185Asp, Meth186Tyr, His187Ala, Val188Arg

*The same mutation was found during the original sequencing of the VLD1 *EcoR*I region but did not rescue VLD1 very late gene expression as determined by marker rescue experiments (Merrington *et al.*, 1996).

**Mutation could only be confirmed with the forward primer, while the reverse primer and a different primer pair covering the same region in *pk-1* did not amplify a sufficient amount of DNA required for Sanger sequencing.

Cysteine residues are polar non-charged amino acids containing a –SH side group and can therefore build inter- or intramolecular disulphide bonds (reviewed by Marino and Gladyshev, 2012). As serines are the most similar amino acids to cysteine having an –OH side chain, cysteine residues inside AcMNPV LEF-2 were exchanged with serine residue by crossover PCR (Allen, 2006). However, a transfer vector encoding for LEF-2^{C165S} could not be produced (Allen, 2006). Viruses were generated by co-transfection with linearised Ac Δ lef-2 DNA and the appropriate transfer vector followed by one round of plaque purification. However, AcLEF-2^{C150S} did not produce any plaques indicating that this amino acid change might be lethal for protein function and therefore virus replication (Allen, 2006). Sanger sequencing of the recombinant viruses revealed a successful amino acid change of two of the most highly conserved LEF-2 cysteine residues resulting in the viruses: AcLEF-2^{C84S} and AcLEF-2^{C146S}. In addition, AcLEF-2^{C88S} was generated (Source BioScience, data not shown). The LEF-2 cysteine 88 is not conserved (Table 4.2; supplementary Fig. S 4.1), is not part of a LEF-2 secondary structure element (black box,

Fig. 4.7) and did only have a moderate prediction to affect protein function (data not shown; Kelly *et al.*, 2015). Nevertheless, this virus containing a mutation inside a non-conserved LEF-2 cysteine residue was included as a control for the analysis of the highly conserved residues.

Both *Sf9* and *T. ni* Hi5 cells infected with either AcLEF-2^{C84S}, AcLEF-2^{C88S} or AcLEF-2^{C146S} accumulated the very late protein P10 to the same level as wt-infected cells from 48-96 h p.i. (Fig. 4.11) indicating that the three mutations inside LEF-2 C84S, C88S and C146S were not required for very late gene expression. As all three virus mutants were generated using AcΔlef-2 as a backbone, their effects on *polh* expression could not be analysed.

To investigate the effects of the highly conserved LEF-2 cysteine residues on *polh* expression, new cysteine mutant viruses encoding for *polh* were generated (Fig. 4.12). Furthermore, to increase the chances to generate the recombinant viruses, the LEF-2 cysteine residues were replaced by an alanine residue. Alanine is a small nonpolar amino acid containing a –CH₃ side group and is thereby one of the most simplest amino acids. It is moreover, classically used for mutagenesis studies (reviewed by Morrison and Weiss, 2001). The following transfer vectors could be created and verified by Sanger sequencing (Source BioScience, data not shown): pBS.SK+CB1^{LEF-2,C84A}, pBS.SK+CB1^{LEF-2,C143A}, pBS.SK+CB1^{LEF-2,C146A}, pBS.SK+CB1^{LEF-2,C150A}, pBS.SK+CB1^{LEF-2,C165A}. Viruses were generated by co-transfection of linearised AcΔlef-2 DNA of *Sf9* cells with the appropriate transfer vector followed by one round of plaque purification.

Plaques from AcLEF-2^{C84A} and AcLEF-2^{C165A}-infected cells were amplified and the amino acid change inside LEF-2 of the recombinant viruses were verified by Sanger sequencing (Source BioScience, data not shown). Infection of *Sf21* cells with AcLEF-2^{C143A}, AcLEF-2^{C146A} and AcLEF-2^{C150A} did not produce any plaques and cells did not show any cpe (data not shown). Therefore, for those viruses attempts were made to passage the virus from the initial co-transfection twice in monolayer culture (P2 virus stock). The DNA was extracted from the media of the P2 stock and screened for *gp64* and *lef-2* as described before for AcLEF-2^{A154T}. However, neither *gp64* nor *lef-2* could be amplified from extracted DNA by PCR for any of the recombinant virus infected cells although both were amplified when cells were infected with wt (data not shown). This indicated that the LEF-2 mutations C142A, C146A and C150A were non-permissive for protein function and virus replication.

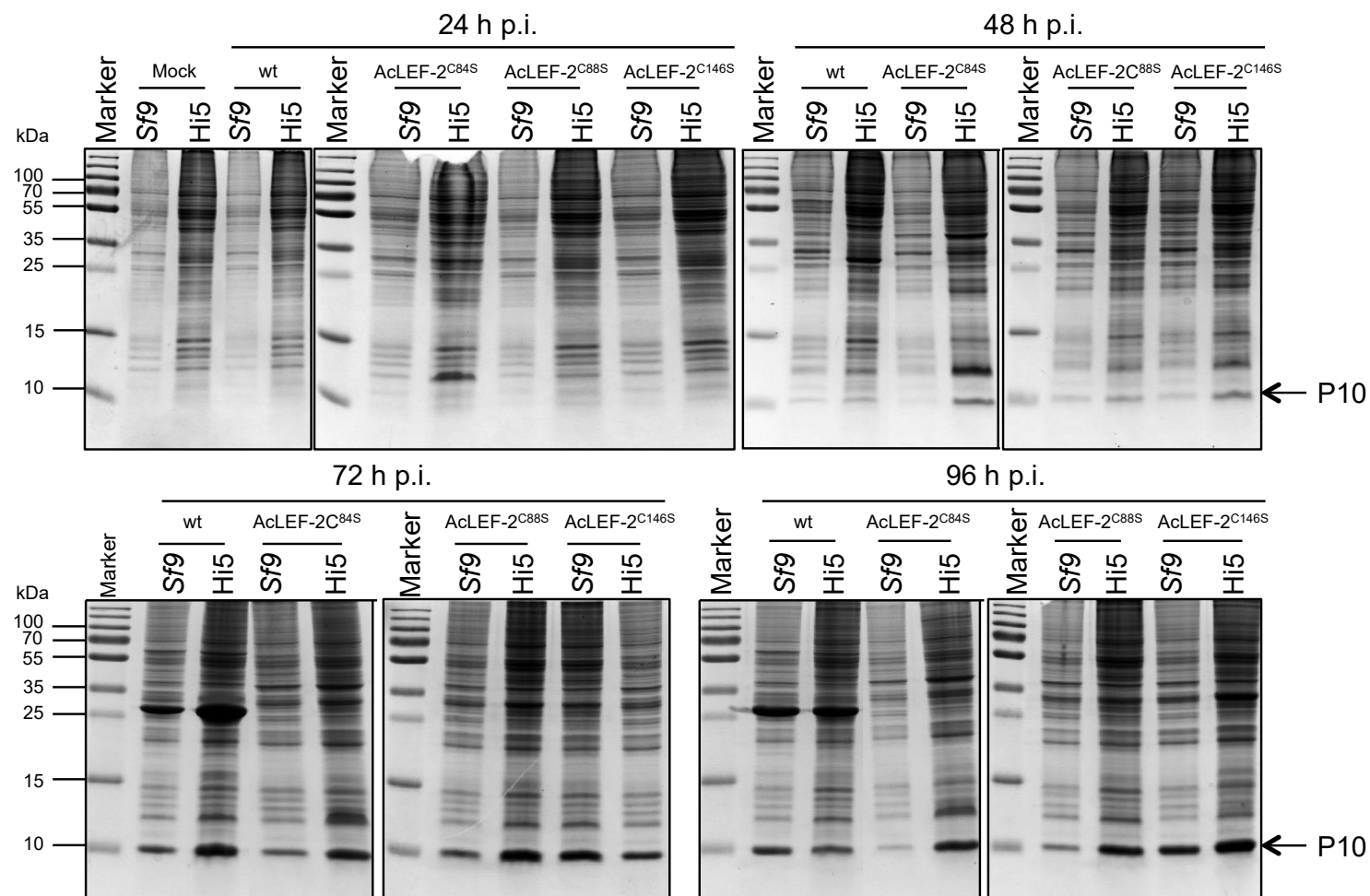


Figure 4.11 Time course of AcLEF-2^{C84S}, AcLEF-2^{C88S} and AcLEF-2^{C146S} infected cells

Sf9 and *T. ni* Hi5 (Hi5) cells were infected with AcLEF-2^{C84S}, AcLEF-2^{C88S} or AcLEF-2^{C146S} at MOI 3 to determine their effects on viral very late gene expression. As controls cells were infected with wild-type (wt) AcMNPV or were mock-infected. Cells were harvested every 24 h over a period from 24-96 h p.i. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrow highlights accumulated P10.

When *Sf9* or *T. ni* Hi5 cells were infected with either AcLEF-2^{C84A} or AcLEF-2^{C165A} both P10 and polyhedrin accumulated at levels similar to wt from 48 h p.i. until 96 h p.i. (Fig. 4.13). However, it could be argued that less polyhedrin accumulated in *Sf9* cells after AcLEF-2^{C184A} infection at 72 h p.i. and in AcLEF-2^{C84A} and AcLEF-2^{C165A}-infected *T. ni* Hi5 cells at 96 h p.i. This suggested that the LEF-2 mutations C84A and C165A may have only a minor impact on *polh* but not on *p10* expression. Moreover, virus infection with either of the mutants led to the accumulation of more viral very late protein in cells than infection with Ac Δ lef-2^{rescue} (Fig. 4.13). However, while the virus LEF-2 cysteine mutants were plaque purified, Ac Δ lef-2^{rescue} was not; providing additional evidence that the Ac Δ lef-2^{rescue} stock most likely contains background contamination of the parental virus.

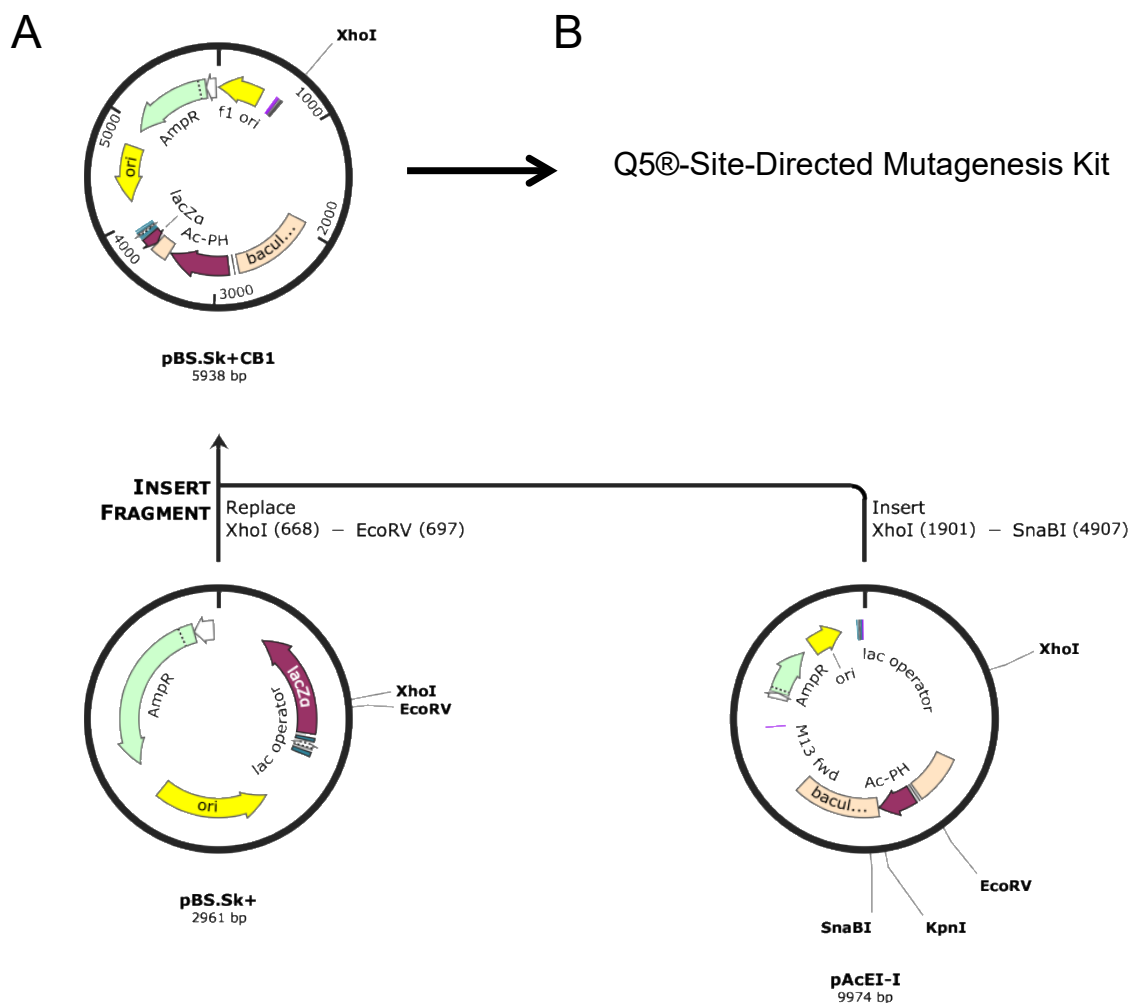


Figure 4.12 Generation of LEF-2 cysteine replacement mutants

A) The *XhoI*-*EcoRV* fragment of pBS.SK+ was replaced with the *XhoI*-*SnaBI* fragment of pAcEI-I containing *lef-2* and its upstream region including *polh*. Thereby pBS.SK+CB6 was generated and utilised as a backbone for further cloning. The DNA sequence was verified by Sanger sequencing (Source Bioscience, data not shown). Graphic was generated using SnapGene® version 4.1.7. B) Nucleotides encoding for cysteine amino acids were substituted inside *lef-2* by utilising 5' back-to-back primers amplifying the entire vector. Mutagenesis was performed using Q5®-Site-Directed Mutagenesis Kit according to manufacturer's instructions (NEB®). Viruses were generated by co-transfection of linearised Ac Δ lef-2 DNA of *Sf9* cells with the appropriate transfer vector followed by one round of plaque purification.

4.4 The role of AcMNPV LEF-2 in virus DNA replication

4.4.1 Establishing a multiplex-qPCR assay to analyse viral replication

As *lef-2* is required for AcMNPV replication (Kool *et al.*, 1994; Lu and Miller, 1995), the effect of the various AcLEF-2 mutants (described in 4.3) was investigated on intracellular viral DNA levels using qPCR. Therefore, an appropriate qPCR assay was developed.

As baculovirus replication starts about 5 h p.i., reaches a maximum at 18 h p.i. and is completed by 24 h p.i. (Tjia *et al.*, 1979), both virus and host DNA was extracted at 24 h p.i. for qPCR analysis (by DNeasy Blood and Tissue Kit, QIAGEN®).

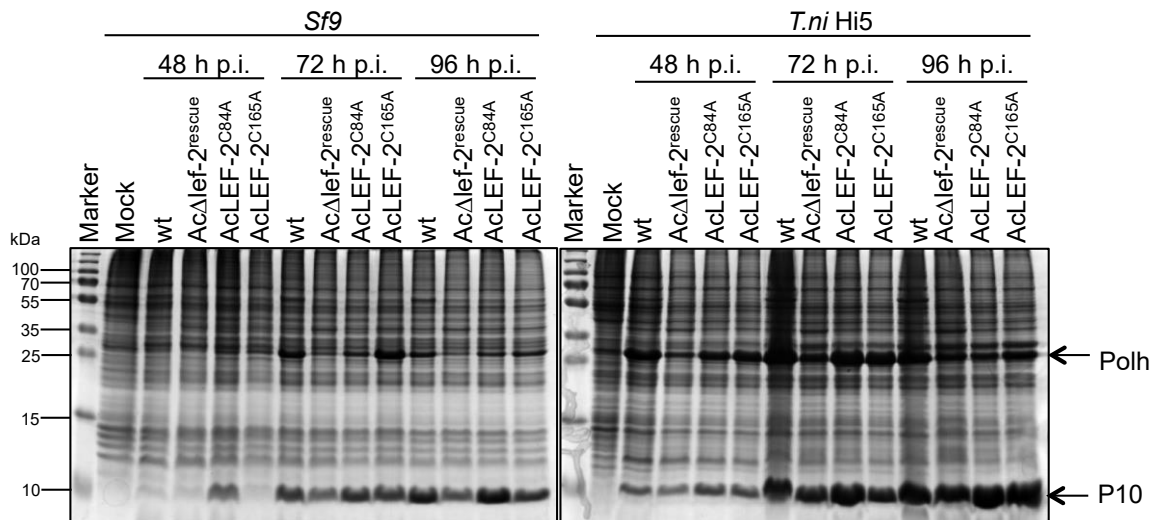


Figure 4.13 Time course analysis of AcLEF-2^{C84A} and AcLEF-2^{C165A} infected cells

Sf9 and *T. ni* Hi5 cells were infected with AcLEF-2^{C84A} and AcLEF-2^{C165A} at MOI 3 to determine their effects on viral very late gene expression. As controls cells were infected with wild-type (wt) AcMNPV, AcΔlef-2^{rescue} or were mock-infected. Cells were harvested every 24 h over a period from 48-96 h p.i. Proteins were separated by a 15% SDS-PAGE and analysed by Coomassie staining. Arrows highlight polyhedrin (Polh) and P10.

In general, two different qPCR chemistries are available either based on the SYBR Green dye or TaqManTM Probe. During the qPCR reaction, the SYBR Green dye binds non-specifically to any dsDNA. It exhibits little fluorescence when it is free in solution but fluorescence increases dramatically when it binds dsDNA. Hence, the fluorescent signal is proportional to the amount of dsDNA and will increase as the target DNA is amplified. On the contrary, the TaqManTM probe oligonucleotide is labelled with a 5' reporter fluorophore and a 3' quencher. The probe specifically binds to the target sequence. During DNA extension the probe is partially displaced, the reporter is cleaved and the reporter fluoresces, while the fluorescence is quenched when not binding to the specific target. Therefore, the fluorescent signal is proportional to the amplified product. The TaqManTM probe chemistry provides several advantages: it is highly specific; it has a very high

signal-to-noise ratio; and moreover, it allows a multiplex reaction. A multiplex reaction is the amplification of more than one target in a single reaction tube. Less starting material is required and the throughput is increased, which reduces reagent costs. Most importantly, however, the number of false negative results is reduced as the control target (endogenous control) is amplified within the same sample (Applied Biosystems Protocol).

Although establishment of a multiplex qPCR assay is more challenging, it was decided to design a dualplex qPCR assay using *actin* as an endogenous control. As a target to measure viral replication it was decided to utilise *gp64*. The viral glycoprotein GP64 is incorporated into the membrane of BV exclusively and is the limiting factor for BV production (Oomens and Blissard, 1999).

The *gp64* and *actin* primer pairs were designed (Materials and Methods Table 2.6) and their specificity was examined by a melt curve analysis. The highly specific melt curve analyses were performed using the SYBR Green dye initially to determine any amplification of dsDNAs during the qPCR reaction. Furthermore, it analysed how much and how many products are being generated. This was achieved by increasing the temperature in small increments and monitoring the fluorescent signal at each step after amplification; as the dsDNA denatures, the fluorescence signal decreases. A melt curve is plotted as the negative first derivative of fluorescence against temperature ($-dF/dT = -R_n$), whereby the maximum is defined as 50% dissociation. Optimal would be a single peak at a temperature higher than 80°C and nothing in the non-template (negative) control (Applied Biosystems Protocol). The melt curve for *gp64* and *actin* primer pairs are shown in Fig. 4.14.

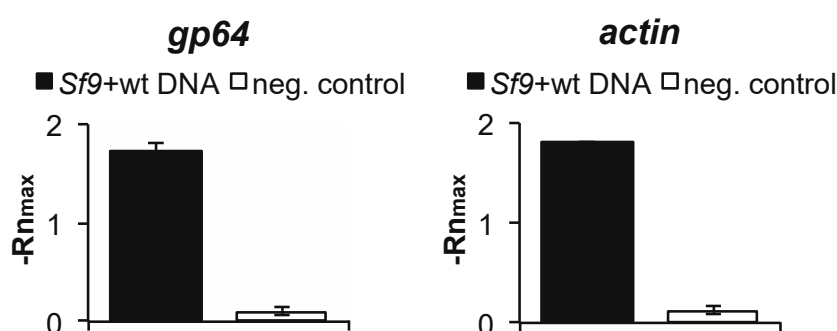


Figure 4.14 Specificity investigation of the primer pairs qPCR *gp64* F2/*gp64* R4 and qPCR *actin* 13/*actin* 14

The qPCR *gp64* primer pair (F2/R4; 600 nM each) and *actin* primer pair (13/14; 900 nM each) were used to amplify 0.1 µg DNA extracted from wild-type AcMNPV-infected *Sf9* cells (*Sf9*+wt DNA) at 24 h p.i. by qPCR followed by a melt curve analysis. Shown is the first negative derivative of the fluorescence against the temperature, which is an indication of how much of the product was amplified. Error bars represent standard deviations (n=3; technical replicates).

The design of the probes was highly dependent on the technical details of the qPCR machine utilised (Applied Biosystems 7000/7500, e.g. available filter) as well as the recommendations for the dual probes used (Eurofins Genomics). Taking everything into account it was decided to design probes with the following reporters and quenchers: 5'FAM-*gp64*-3'BHQ1 and 5'TAM-*actin*-3'BHQ2 (Materials and Methods Table 2.6). The absorbance of the FAM reporter is 495 nm with a maximal emission at 520 nm, while the TAMRA (TAM) reporter absorbance is at 544 nm with a maximal emission at 576 nm. The quenching maximum of BHQ1 and BHQ2 is at 535 nm and 579 nm, respectively.

The reaction conditions for the primers and the probes were optimised to determine the minimal concentrations required for the qPCR reaction, which is known as primer limiting (Applied Biosystems Protocol). The limiting concentrations of probe/primers are used to amplify the more abundant species, and thereby “turning off” the amplification as soon as the C_T has been established (Fig. 4.15). The C_T value is the fluorescence threshold cycle during a qPCR reaction by which the fluorescent signal is first significantly higher than the background. It is inversely proportional to the amount of the DNA template or in simple words: the more DNA, the lower the C_T .

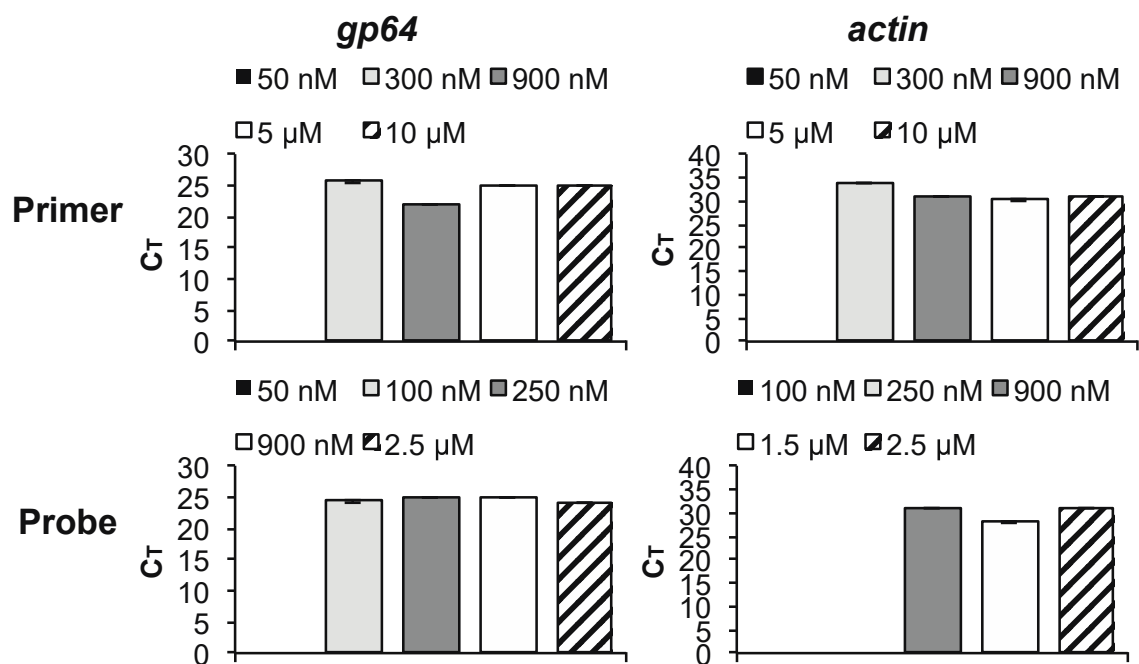


Figure 4.15 Primer and probe titration for a multiplex qPCR

The qPCR *gp64* primer pair (F2/R4) and *actin* primer pair (13/14) were titrated at the indicated concentrations by amplifying 0.1 μg DNA extracted from wild-type AcMNPV-infected *Sf9* cells (*Sf9*+wt DNA) at 24 h p.i., whereby 2.5 μM of the respective probe was utilised during the reactions. The 5'FAM-*gp64*-3'BHQ1 and the 5'TAM-*actin*-3'BHQ2 probe were titrated at the indicated concentrations by amplifying 0.1 μg DNA extracted from wt-infected *Sf9* cells (*Sf9*+wt DNA) at 24 h p.i., whereby the limiting primer concentrations were utilised during the reacting. The C_T values of the negatives controls were not detected. Error bars represent standard deviations (n=3; technical replicates).

Afterwards, the qPCR amplification efficiency was optimised (Fig. 4.16). Generally, three requirements are necessary for a highly sensitive qPCR assay: a) Consistency between replicates; b) linear standard curve (measured by Pearson's correlation coefficient with $R^2 > 0.98$); and c) high amplification efficiency (90-105%). Additionally for the statistical analysis of multiplex assays, the efficiency for the amplicons must not differ by more than 5% (Applied Biosystems Protocol). Therefore, the limiting primers and probes concentrations were initially used to analyse the qPCR amplification efficiency. If the amplification efficiency was not in the required range, primer and probe concentrations were increased in small steps (data not shown).

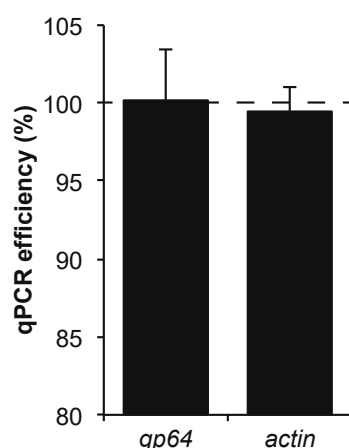


Figure 4.16 Optimisation of amplification efficiency for a multiplex qPCR

0.01 µg, 0.1 µg, 1 µg *Sf9*+wt DNA were amplified either using 600 nM qPCR *gp64* F2 and R4 primers or 900 nM qPCR *actin* 13 and 14 and either 600 nM 5FAM-*gp64*-3'BHQ1 probe or 900 nM 5FAM-*actin*-3'BHQ2 by qPCR to identify the minimum primer and probe concentration required for a highly sensitive qPCR (95-105% qPCR efficiency). The C_T values of the negative controls were not detectable. Error bars represent standard deviations (n=3; technical replicates).

The last step was to validate the designed multiplex assay by the comparison of the C_T values of both single- and multiplex setups in the same plate; which should not be different (Fig. 4.17). In total seven primer pair combinations for *gp64* and eleven primer pair combinations for *actin* were tested (data not shown; Material and Methods Table 2.6) before the primer pairs qPCR *gp64* F2/*gp64* R4 and qPCR *act13*/*act14* were chosen for the reasons discussed above (Fig. 4.14-4.17). In the final protocol qPCR multiplex protocol, 600 nM qPCR *gp64* F2, *gp64* R4 and 5FAM-*gp64*-3'BHQ1 probe and 900 nM *actin* 13, *actin* 14 and 5FAM-*actin*-3'BHQ2 probe were utilised using 500 ng template DNA.

One last comment should be made in regards of the design of the multiplex qPCR to analyse viral replication: The assay was optimised for *Sf9* cells and may not work in virus-infected *T. ni* Hi5 cells. The reason why *Sf9* were chosen over *T. ni* Hi5 cells was to

analyse how virus replication may affect BV production, which is typically performed in *Sf9* cells.

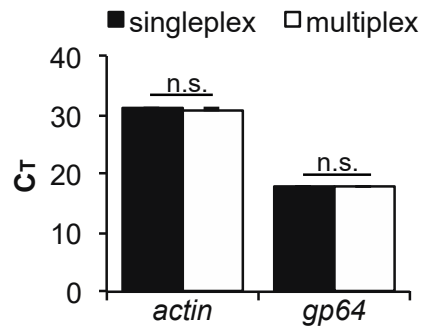


Figure 4.17 Comparison of qPCR single- vs. multiplex set up

500 ng *Sf9*+wt DNA were amplified either using 600 nM qPCR *gp64* F2 and R4 primers or 900 nM qPCR *actin* 13 and 14 and either 600 nM 5'FAM-*gp64*-3'BHQ1 probe or 900 nM 5'FAM-*actin*-3'BHQ2 by qPCR with in single or in multiplex reaction to compare the C_T values. Significance was calculated using Student's t-test with p<0.05; n.s.=not significant. Error bars represent standard deviations (n=3; technical replicates).

4.4.2 The effect of LEF-2 mutagenesis on viral replication

Viral and cellular DNA of wt or *lef-2* recombinant virus-infected (viruses from 4.3) *Sf9* cells (MOI 3) was extracted at 24 h p.i. (by DNeasy Blood and Tissue Kit, QIAGEN®) and the relative intracellular viral DNA amount was analysed by comparative qPCR ($-2^{\Delta\Delta C_T}$). The comparative qPCR analysis involved two normalisation steps. First, the C_T of the target (*gp64*) was normalised against the C_T value of the endogenous control (*actin*) resulting in ΔC_T . Second, the ΔC_T values of the recombinant viruses were normalised against the ΔC_T value of the wt sample resulting in $\Delta\Delta C_T$. Afterwards, the relative intracellular viral DNA was calculated by using the formula $-2^{\Delta\Delta C_T}$ (Livak and Schmittgen, 2001).

Infection of *Sf9* cells with VLD1 reduced intracellular viral DNA by 91% (Fig. 4.18), while both AcLEF-2^{D178N, polh-} and AcLEF-2^{D178N} did not significantly affect viral replication when compared to wt-infected (Fig. 4.18). In addition, relative intracellular viral DNA of AcLEF-2^{D178N, polh-} and AcLEF-2^{D178N} infected cells did not differ (Fig. 4.18). This further indicated that the mutations in VLD1 and LEF-2^{D178N} did not cause the same phenotype. When cells were infected with the VLD1 rescue viruses, either VLD1^{AcApolh} or VLD1^{EcoR-I} virus replication did not significantly vary compared to wt or AcUW1.*lacZ*-infected cells, respectively (Fig. 4.18). Moreover, the relative intracellular viral DNA in wt and AcUW1.*lacZ* cells was not different (Fig. 4.18).

Infection of *Sf9* cells with AcLEF-2^{C88S} or AcLEF-2^{C146S} reduced relative intracellular viral DNA compared to wt-infected cells by 74% and 94%, respectively (Fig. 4.23). Surprisingly, viral replication of AcLEF-2^{C165A} increased viral DNA replication more than 3-fold

compared to wt-infected cells (Fig. 4.18). Infection of AcLEF-2^{C84S} and AcLEF-2^{C165A} did not have a significant effect on intracellular DNA (Fig. 4.18). Overall, this suggested that different regions and amino acids of LEF-2 are crucial for viral replication.

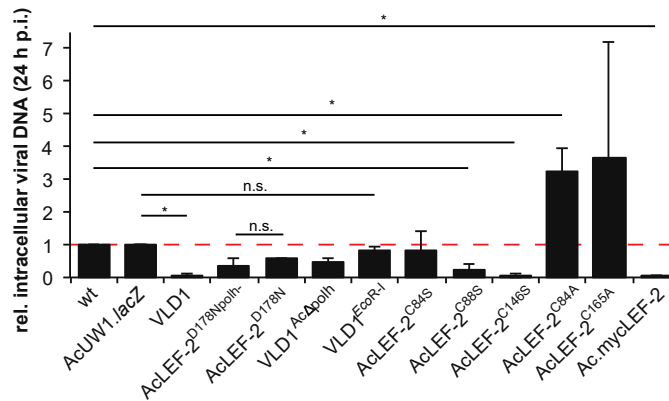


Figure 4.18 The effect of AcMNPV *lef-2* mutagenesis on viral replication in *Sf9* cells

Intracellular viral DNA of various *lef-2* mutants at 24 h p.i. were determined by a multiplex qPCR assay analysing intracellular DNA levels of *gp64* and *actin* as a normaliser in *Sf9* cells. Relative intracellular viral DNA of *lef-2* mutants was normalised to wt intracellular viral DNA using $-2^{\Delta\Delta CT}$ assay. Dashed line (red) indicates rel. intracellular viral DNA of wild-type (wt) AcMNPV-infected cells. Significance was analysed by ANOVA with $p < 0.05$, n.s.=not significant. N.s. is only shown when not compared to wt control. Error bars represent standard deviations ($n=3$; biological replicates).

4.5 The role of AcMNPV LEF-2 on BV production

As some of the LEF-2 virus mutants affected viral replication, their effect on BV production was examined in more detail. For this, *Sf9* cells (in shake culture) were infected with the virus of interest at MOI 0.1 and BV production was determined by BaculoQUANT™ qPCR at 5 d p.i. (OET Ltd.; Hitchman *et al.*, 2007). Infection with VLD1 reduced BV production $\sim 0.5 \log_{10}$ compared to wt-infected cells, while BV production of either AcLEF-2^{D178N, polh-} or AcLEF-2^{D178N}-infected cells was neither different between each other nor to wt-infected cells (Fig. 4.19A). This further suggested that the phenotype of AcMNPV LEF^{D178N} varied compared to VLD1. Moreover, BV production of AcLEF-2^{A154T, D178N} and AcLEF-2^{A154T, D178N, polh-} did not vary compared to AcΔ*lef-2*^{rescue} infected cells. This indicated that LEF-2^{A154T, D178N} did not affect BV production. However, the BV titre of AcΔ*lef-2*^{rescue}-infected cells was significantly lower than wt-infected cell (Fig. 4.19B). In addition, when VLD1 was rescued with either AcΔ*polh* or the AcMNPV *EcoR-I* fragment, BV titres returned back to wt or AcUW1.*lacZ* BV titres, respectively (Fig. 4.19C). Moreover, BV production in wt and AcUW1.*lacZ* was not different (Fig. 4.19A, C). In addition, BV titres of AcLEF-2^{C84S}, AcLEF-2^{C146S} and AcLEF-2^{C84A}-infected cells was reduced by $\sim 0.5 \log_{10}$, $\sim 1.5 \log_{10}$ and $\sim 0.3 \log_{10}$, respectively (Fig. 4.19A, D). Overall, this suggested that the certain mutants of the AcMNPV *lef-2* could reduce BV production of infected cells.

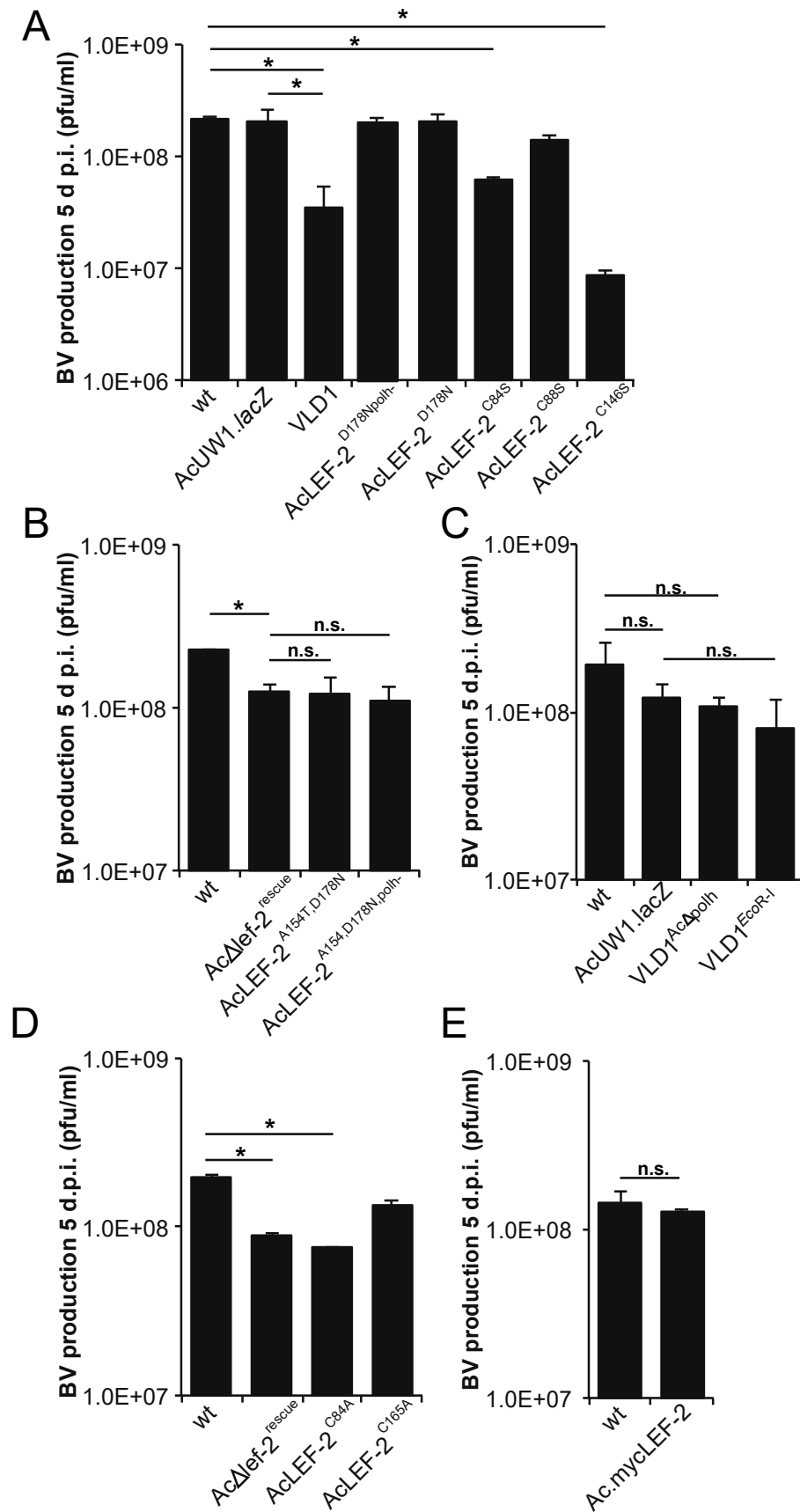


Figure 4.19 The effect of AcMNPV *lef-2* mutagenesis on BV production in Sf9 cells

A-D) Sf9 cells in shake cultures were infected with the indicated virus at MOI 0.1. At 5 d p.i. the virus titres were determined by BaculoQUANT™ qPCR (OET Ltd.; Hitchman *et al.*, 2007). Significance was analysed by ANOVA with $p < 0.05$, n.s.=not significant. Error bars represent standard deviations ($n \geq 3$; biological replicates).

4.6 AcMNPV LEF-2 transcription, translation and localisation

4.6.1 LEF-2 transcription

To determine the *lef-2* transcription profile, data from a RNA-sequencing experiment (Chapter 6) were taken. In AcMNPV C6-infected *T. ni* Hi5 cells (MOI 5) transcription of *lef-2* was detected from 2 h p.i., which was the earliest analysed time point p.i. (Fig. 4.20). Transcription of *lef-2* rapidly increased until 12 h p.i. and subsequently decreased reaching a plateau between 18-24 h p.i. highlighting a role of *lef-2* in virus replication. From 24 h p.i., *lef-2* transcription by wt-infected *T. ni* Hi5 cells slightly increased until the last time point analysed at 48 h p.i.

Interestingly, in a different system, the *lef-2* transcription profile varied to that analysed in this thesis. When the *T. ni* Tnms42 cells (Materials and Methods Table 2.1; Chapter 5 for details on this cell line) were infected with a different virus isolate AcMNPV E2 at MOI 10, *lef-2* transcription slowly increased until 36 h p.i. and subsequently started to decrease until the last analysed time point p.i. at 48 h p.i. (Fig. 4.20; data taken from Chen *et al.*, 2013).

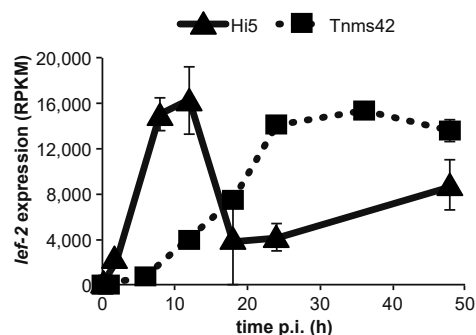


Figure 4.20 *lef-2* transcription profile in AcMNPV-infected *T. ni* cells

Normalised *lef-2* gene expression values (RPKM) of AcMNPV C6 infected *T. ni* Hi5 (Hi5) cells (MOI 5) and normalised gene expression values of AcMNPV E2 infected Tnms42 cells (MOI 10) were plotted against time of infection. Data for virus-infected *T. ni* Hi5 cells were taken from the Illumina® RNA-sequencing data set analysed in Chapter 6. Data for virus-infected Tnms42 cells were taken from Chen *et al.*, 2013. Error bars represent standard deviations (n=3; technical replicates).

4.6.2 LEF-2 antibody

So far, the mutations of LEF-2 have been analysed in the context of viral very late gene expression, viral replication and BV production. However, another interesting aspect would be to determine how these mutations influence protein localisation inside virus-infected cells. Therefore, a polyclonal LEF-2 antibody was generated by the immunisation

of New Zealand Rabbit against the LEF-2 immunogenic sequence: CLDKKATYLIDPDD. This sequence corresponded to amino acid positions 17-30 at the LEF-2 N-terminus. The antibody was recovered by affinity peptide purification (GeneScript®).

The LEF-2 antibody was tested after proteins of wt-infected *Sf9* and *T. ni* Hi5 cell lysates were separated by a SDS-PAGE and transferred onto a nitrocellulose membrane. The generated antibody recognised a ~35 kDa protein from 6 h p.i., which accumulated until 96 h p.i. in wt-infected *Sf9* cells but not in mock-infected cells. The same protein was detected by the antibody in wt-infected *T. ni* Hi5 cells (data not shown). However, the AcMNPV LEF-2 proteins was predicted to have a size of 23.9 kDa (Ayres *et al.*, 1994). Moreover, *Sf21* cells infected with AcMNPV that was 5'myc-tagged (Aclef-2.cmyc5') accumulated ~25 kDa protein after protein separation by SDS-PAGE and western blotting against c-MYC. This protein was detected as early as 4 h p.i. and accumulated at least until 24 h p.i. in virus-infected cells (R. D. Possee, personal communication). This suggested that the generated antibody might recognise another viral protein instead of LEF-2.

4.6.3 Generation of Acmyc.LEF-2

To confirm that the LEF-2 antibody recognised a nonspecific protein in virus-infected cell lysates, the 5' *lef-2* was *c-myc* tagged. The c-MYC tag comprises a 12 amino acid sequence that can be specifically recognised by an antibody directed against this sequence (Fig. 4.21B). Unfortunately, the original Aclef-2.cmyc5' was not available anymore (R. D. Possee, personal communication). Hence, the 5'*c-myc*-tagged *lef-2* AcMNPV was regenerated to that in the original work. In the original work a 3'*c-myc*-tagged *lef-2* could not be generated as is probably interfered with protein folding or activity (R. D. Possee, personal communication). Therefore, a transfer vector (pBS.SK+CB6) was generated, which encoded a C-terminal c-MYC tagged LEF-2 (Fig. 4.21). The nucleotide sequence was verified by Sanger sequencing (Source BioScience, data not shown).

Insect cells were co-transfected with the linearised AcΔlef-2 bacmid DNA and the transfer vector pBS.SK+CB6. The resultant virus was plaque purified generating *polh*-positive virus. However, Sanger sequencing of the virus revealed that the *c-myc* tag had been lost although the sequence of *lef-2* was not affected. In addition, polyhedrin still accumulated in virus-infected cells (data not shown). Even when the virus was not plaque purified, the *c-myc* tag had been lost indicating that the protein tag might interfere with protein function (verified by Sanger sequencing, data not shown). Therefore, it was decided to co-transfect the transfer vector with circular, non-digested AcΔlef-2 DNA although this increased the risk of generating a mixed population of virus. When AcΔlef-2 DNA was not digested by

SrfI, Ac.mycLEF-2 was generated as verified by Sanger sequencing (Source BioScience, data not shown).

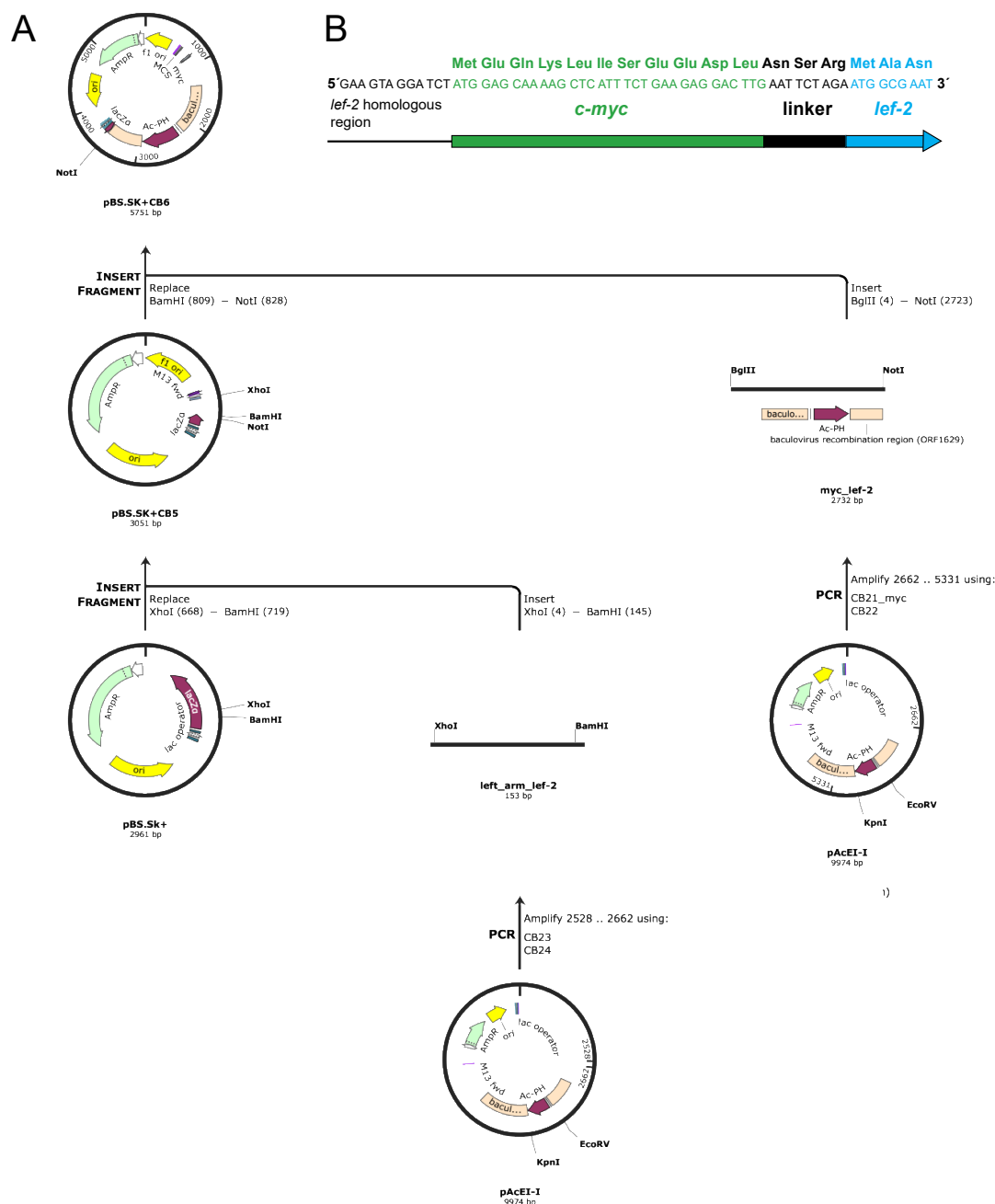


Figure 4.21 Generation of an Ac.mycLEF-2 transfer vector and genomic organisation of Ac.mycLEF-2

A) Ac.mycLEF-2 was generated by two cloning steps. First, the left homologous arm of *lef-2* was amplified by PCR using the prime pair CB23 and CB24 and pAcEI-I as a template. Thereby a *XhoI* and *BamHI* restriction digestion site was added at the 5' and 3' end of the DNA amplification product, respectively. Subsequently, the *XhoI*-*BamHI* fragment of pBS.SK+ was replaced with the DNA sequence of the *lef-2* homologous arm generating pBS.SK+CB5. Second, the 5' *lef-2* was *c-myc*-tagged by the amplification of pAcEI-I with the primer CB21 and CB22. At the same time a *BamHI* and *NotI* restriction digestion sites were added at the 5' and 3' end of the DNA amplification product. The myc.*lef-2* DNA fragment was inserted into pBS.SK+CB5 by replacing its *BamHI*-*NotI* region and thereby generating pBS.SK+CB6. The DNA sequence of each cloning step was verified by sequencing (data not shown). Graphic was generated using SnapGene® version 4.1.7 B) The *c-myc* (green) coding region was inserted at the 5' end of *lef-2* (blue), which are separated by a linker region (black). The encoded amino acids are shown in bold above the nucleotide sequence.

It was then examined how the potential mixed Ac.mycLEF-2/Ac Δ lef-2 population might affect virus replication and BV production. While intracellular DNA levels of Ac.mycLEF-2 infected *Sf9* cells were reduced by 96% (Fig. 4.18), BV production was not different compared to wt-infected cells (Fig. 4.19E). This indicated that virus replication was delayed in Ac.mycLEF-2-infected cells.

However, after protein separation of Ac.mycLEF-2-infected *Sf9* cell lysates by SDS-PAGE and western blotting using a c-MYC antibody, no specific protein accumulations could be detected (data not shown). However, a successful protein transfer onto the nitrocellulose membrane was verified by Ponceau S staining and moreover, a specific band was detected at the expected size after running the cell lysates of a positive control virus expressing a 31 kDa c-MYC-tagged protein (data not shown). This indicated that *c-myc* in Ac.mycLEF-2-infected cells was not expressed. Therefore, attempts to localise LEF-2 in virus-infected cells were not pursued further.

4.7 Discussion

The aim of this chapter was to analyse the potential dual role of AcMNPV LEF-2 in both virus replication and very late gene expression by a series of mutagenesis studies (Fig. 4.22; Table 4.4). A bioinformatics approach confirmed that LEF-2 may have a dual molecular function by having primase activity and by binding of 4Fe-4S clusters (Fig. 4.1; Hamp *et al.*, 2013). The 4Fe-4S clusters have been demonstrated to regulate gene expression at both transcriptional and translational level (reviewed by Johnson *et al.*, 2005).

Table 4.4 The effect of AcMNPV *lef-2* mutagenesis on viral very late gene expression, virus replication and BV production: Summary

Virus	Very late gene expression	Viral replication (24 h p.i.)	BV production (5 d p.i.)
AcUW1. <i>lacZ</i>	0	0	0
VLD1	-	-	-
AcLEF-2 ^{D178N, polh-}	0	0	0
AcLEF-2 ^{D178N}	0	0	0
AcLEF-2 ^{A154T, D178N, polh-}	0	?	0
AcLEF-2 ^{A154T, D178N}	0	?	0
AcVLD1 ^{AcApolh}	0	0	0
AcVLD1 ^{EcoRI}	0	0	0
AcLEF-2 ^{C84S}	0	0	-
AcLEF-2 ^{C88S}	0	-	0
AcLEF-2 ^{C146S}	0	-	-
AcLEF-2 ^{C84A}	0	+	-
AcLEF-2 ^{C165A}	0	0	0
Ac Δ lef-2 ^{rescue}	-	?	-
Acmyc.LEF-2	?	-	0
Potentially lethal mutations:			
AcLEF-2 ^{A154T} , AcLEF-2 ^{C143S} , AcLEF-2 ^{C150S} , AcLEF-2 ^{C165S} , AcLEF-2 ^{C143A} , AcLEF-2 ^{C146A} , AcLEF-2 ^{C150A}			

*0 no significant changes to control virus; - reduced compared to control virus; + increased compared to control virus; ? not analysed

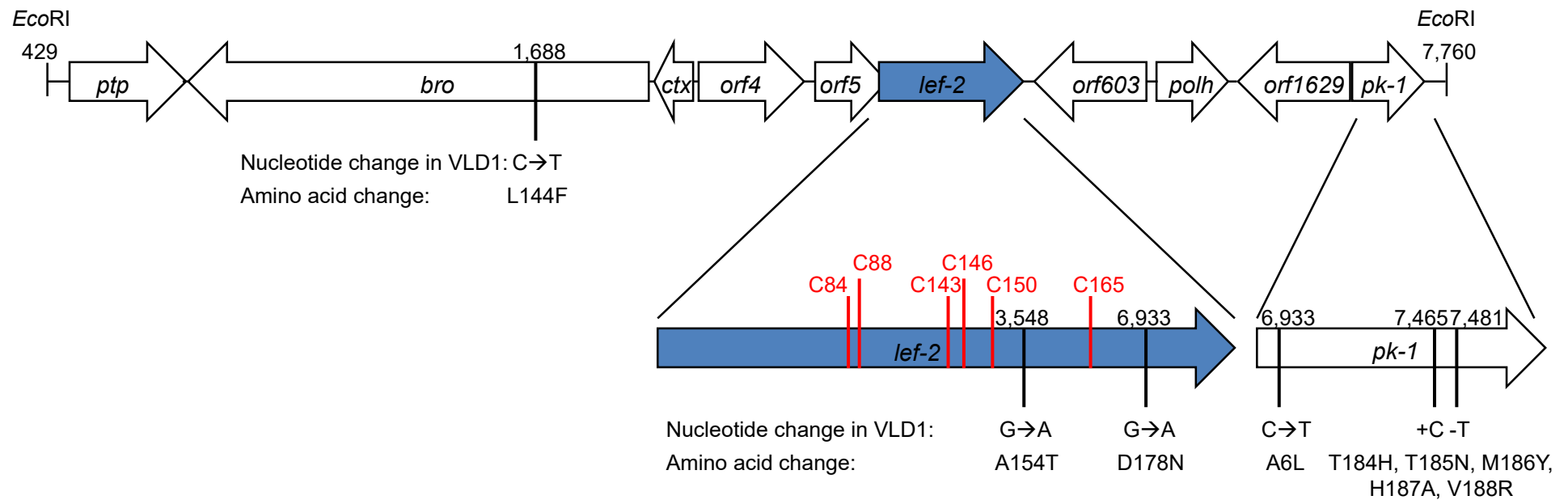


Figure 4.22 Genomic organisation of AcMNPV *EcoRI* region and mutations analysed

The 7.3 k bp AcMNPV *EcoRI* fragment is represented relative the AcMNPV sequence (numbers above arrows, Ayres *et al.*, 1994). The orfs are shown as arrows, which indicate directions of transcription. Nucleotide changes found in VLD1 are indicated as black vertical lines with the relative genome sequence above and the encoded amino acid change below. Red lines indicate mutagenesis studies performed on *lef-2* (blue) encoded cysteine amino acids.

The virus VLD1 is deficient in the activation of very late gene expression in *Sf* cells (Merrington *et al.*, 1996). These results were confirmed in *T. ni* Hi5 cells indicating that VLD1 does not act in a cell line specific manner (Fig. 4.2-4.4). Surprisingly, protein analysis of infected cells with viruses (AcLEF-2^{D178Npolh-} and AcLEF-2^{D178N}) containing the identified amino acid changes responsible for the VLD1 phenotype, still expressed *p10* and *polh* suggesting that LEF-2^{D178N} is not essential for very late gene expression (Fig. 4.4, 4.5). Moreover, neither AcLEF-2^{D178Npolh-} nor AcLEF-2^{D178N} had impaired BV production nor impaired viral replication as observed for VLD1 in *Sf9*-infected cells (Fig. 4.18, 4.19A). Both BV production and intracellular DNA levels of AcLEF-2^{D178Npolh-} and AcLEF-2^{D178N}-infected cells were not significantly different to wt-infected cell (Fig. 4.18, 4.19A). Initially, this seemed to contradict published results, where both VLD1 and AcLEF-2^{D178Npolh-} reduced BV production by ~10-fold but did not affect virus replication (Merrington *et al.*, 1996). However, there were differences in the performances of the assays. While BV production in this study was performed at MOI 0.1 and analysed at 5 d p.i., in the original work a MOI of 5 was utilised and analysis was carried out at 48 h p.i. Virus replication was previously analysed by dot blot analysis and a *DpnI* sensitive replication assay. However, the utilised method in this study was qPCR, which is more sensitive (Fig. 4.18, 4.19A; Merrington *et al.*, 1996).

Reanalysis and resequencing of VLD1 *lef-2* identified a second so far undetected point mutation at AcMNPV genome position 3548 resulting in LEF-2^{A154T} (Table 4.3). However, a virus AcLEF-2^{A154T} could not be generated by co-transfection of linearised Ac Δ lef-2 DNA and the appropriate transfer vector indicating that LEF-2^{A154T} might be crucial for protein function and virus replication (data not shown). Technically, co-transfections do not measure viral replication but the homologous recombination potential. Therefore, to investigate if LEF-2^{A154T} is lethal, this mutation should be introduced into a bacmid system. However, the alignment and *in silico* analysis of LEF-2 supported the conclusion that LEF-2^{A154T} might be important for protein function as this amino acid is highly conserved across all baculoviruses and localises in an α -helix secondary structure (Fig. 4.7; Table 4.2; supplementary CD Fig. S 4.1). It may be further speculated that a combination of the LEF-2 mutations A154T and D178N is acceptable for protein structure and function, while the mutation A154T is non-permissive for the virus. Secondary structures were implied to play a role in the LEF-2 and LEF-1 interaction (Evans *et al.*, 1997). While the LEF-2 interacting domain with the viral primase LEF-1 was determined to be localised close to the LEF-2 N-terminus between amino acids 20-40, the LEF-1 interacting domain could not be identified. It was hypothesised that protein secondary structures would play a role for this interaction (Evans *et al.*, 1997).

The effect of both LEF-2 mutations identified in VLD1 (LEF-2^{A154T,D178N}) on very late gene expression was examined by introducing them into the AcMNPV genome. However, LEF-2^{A154T,D178N} did not effect *p10* nor *polh* expression in virus-infected cells suggesting that the mutations mapped to VLD1 *lef-2* were not essential for very late gene expression (Fig. 4.9). Even more, these mutations did not affect BV production (Fig. 4.19B). The effect of LEF-2^{A154T,D178N} on virus replication was not analysed. Both BV and very late gene expression of AcLEF-2^{A154T,D178N} and AcLEF-2^{A154T,D178N,polh-}-infected cells were compared to cells infected with Ac Δ lef-2^{rescue}, which was reduced in comparison to wt-infected cells (Fig. 4.9, 4.19B). The Ac Δ lef-2^{rescue} stock potentially consists of a mixed population of Ac Δ lef-2^{rescue}/Ac Δ lef-2 caused by religated or non-digested Ac Δ lef-2 DNA during virus generation. In this population Ac Δ lef-2^{rescue} can act as a helper virus resulting in the replication of Ac Δ lef-2. Moreover, the knock-out of *lef-2* did not completely abolish viral replication (Fig. 4.8; R. D. Possee, personal communication). Transfection of Ac Δ lef-2 bacmid DNA and subsequent analysis by a *DpnI* sensitive DNA replication assay revealed that viral DNA was replicated although reduced and delayed compared to a rescue virus. It was therefore hypothesised that LEF-2 is not required for the initiation of viral replication (Wu *et al.*, 2010). It may be also speculated that host factors may partially compensate for the loss of *lef-2*.

The mutation for the deficiency in VLD1 very late gene expression was identified by marker rescue experiments by the co-transfection of the AcMNPV *EcoR-I* fragment (Merrington *et al.*, 1996). To validate these results a virus VLD1^{*EcoR-I*} was isolated and amplified using the blue colour development after X-Gal staining as a marker after co-transfection of VLD1 with *EcoR-I* DNA. Interestingly, in contrast to previous rescue experiments, polyhedra could not be observed by light microscopy in *Sf21* cells after 3 d p.t.. Even 9 d p.t., polyhedra were not detectable by light microscopy (data not shown; Merrington *et al.*, 1996). Despite using a different media for cell culture maintenance, the cells themselves might have changed over the years resulting in less strong or delayed expression of viral very late genes after co-transfection experiments. However, the *EcoR-I* region could fully rescue the VLD1 phenotype in regards of very late gene expression, viral replication and BV production when compared to the VLD1 parental virus AcUW1.*lacZ* (Fig. 4.10, 4.18, 4.19C). This confirmed that the mutation responsible for the VLD1 phenotypes is present in the *EcoR-I* region.

Resequencing of the VLD1 *EcoR-I* region revealed new undetected mutations inside *pk-1*. At AcMNPV genome position 6933 cytosine was replaced by thymidine resulting in PK-1^{A6V}. Moreover, after AcMNPV genome position 7465 a cytosine residue was inserted and a thymidine residue was deleted after position 7481 causing the following mutations inside PK-1: T184H, T185N, M186Y, H187A, V188R (Table 4.3). Interestingly, the

serine/threonine kinase PK-1 regulates viral very late gene expression by being part of the transcriptional initiation complex that binds to the *polh* transcription initiation sequence and is thereby regulating *polh* transcription (Mishra *et al.*, 2008). Moreover, a *pk-1* ts-mutant was found to have lower levels of viral very late RNA (Fan *et al.*, 1996). It would be interesting to know how a recombinant virus, which recreates the VLD1 *pk-1* mutations would act in regard of viral very late gene expression.

However, even *lef-2* could rescue VLD1 very late gene expression by marker rescue experiments (data not shown; Merrington *et al.*, 1996). Unfortunately, this virus could not be amplified. A combination of different mutations inside VLD1 might be a reason for this. Nevertheless, it may be hypothesised that *lef-2* has additive effects on very late gene expression. A dual mutation of both *p35* and *fp25k* in AcMNPV was observed to contain an additive phenotype (Kelly *et al.*, 2006). While a deletion of the anti-apoptotic gene *p35* results in the induction of apoptosis in virus-infected *Sf* cells, it has no effect in virus-infected *T. ni* cells (Clem *et al.*, 1991; Griffiths *et al.*, 1999). However, a combination of both *p35* and *fp25k* mutations in virus-infected TN-368 cells resulted in a FP phenotype together with plasma membrane blebbing and caspase activation, which are typically signs of apoptosis. When *p35* was rescued, only the FP phenotype remained in virus-infected cells (Kelly *et al.*, 2006).

During the alignment of LEF-2 baculovirus amino acid sequences, it was noted that five cysteine residues at the LEF-2 C-terminus were highly conserved (>96%; Table 4.2; supplementary CD Table S 4.1). Cysteine residues are often important for protein structure and function, also because they are able to form intra- and intermolecular disulphide bonds (reviewed by Marino and Gladyshev, 2012). The viral GP64 is covalently linked by one intermolecular disulphide bond (Cys24-Cys372) and six intramolecular disulphide bonds. While the six GP64 intramolecular disulphide bridge were determined to be critical for virus-mediated pH-triggered membrane fusion, the GP64 intermolecular disulphide bond was not important for this process (Li and Blissard, 2010). However, if LEF-2 is linked by either inter- or intramolecular disulphide bridges this would need to be determined by the generation of crystal structures. Nevertheless, the importance of the LEF-2 cysteine residues during the viral replication cycle was analysed in more detail.

Two different approaches were performed to generate the recombinant LEF-2 cysteine viruses. The cysteine residues were either exchanged by a serine residue in a *polh*-negative virus or by an alanine residue in a *polh*-positive backbone. However, only the following recombinant viruses could be generated by the co-transfection of *SrfI* digested Ac Δ lef-2 DNA with the appropriate transfer vector: AcLEF-2^{C84S}, AcLEF-2^{C88S}, AcLEF-2^{C146S}, AcLEF-2^{C84A}, AcLEF-2^{C165A} (Fig. 4.12; Allen, 2006). This suggested that

LEF-2^{C143S/A}, LEF-2^{C146S}, LEF-2^{C150S/A}, LEF-2^{C165S} may affect protein structure in a way that is crucial and lethal for protein function (Fig. 4.7). Additionally, it may be noted that the replacement with either serine or alanine residues may alter protein structure and therefore protein function differently. As recombinant viruses were generated by co-transfections the lethality of the generated recombinant viruses would need validation in a bacmid system. However, neither of the analysed recombinant cysteine viruses that could be amplified had an effect on viral very late gene expression indicating that those residues are not crucial for *p10* or *polh* expression (Fig. 4.11, 4.13). Interestingly, when the *SalI* region of BmNPV *lef-2* was deleted transactivation from *polyhedrin* promoter constructs was abolished. Apart from LEF-2 D178 this region harbours the C-terminal cysteine rich domain but in addition this domain encoded for other amino acids (96 in total; Sriram and Gopinathan, 1998).

On the other hand, different cysteine viruses had different effects on both intracellular viral DNA level and BV production (Fig. 4.18, 4.19A, D). While the non-conserved LEF-2^{C88S} and the highly conserved LEF-2^{C146S} reduced intracellular viral DNA in virus-infected cells indicating that viral replication is impaired, only BV production of AcLEF-2^{C146S}-infected cells was decreased. This suggested that virus replication in infected AcLEF-2^{C88S} cells was delayed. In addition, BV production of AcLEF-2^{C146S} and AcLEF-2^{C84A}-infected cells was decreased (Fig. 4.19A, D). Interestingly, intracellular viral DNA in AcLEF-2^{C84A}-infected cells was increased, suggesting that the DNA would not get packed into BVs (Fig. 4.19D). The recombinant virus AcLEF-2^{C165A} did not generate any phenotype compared to wt-infected cells (Fig. 4.13, 4.18, 4.19D). In general, the conserved cysteine residues in LEF-2 could play a role in virus replication. Conserved cysteine residues are also crucial for the replication of other viruses such as during the RNA synthesis in Dengue virus infections. The substitution of three cysteine residues with alanine or the deletion of the C-terminus of the Dengue virus non-structural protein 1 (NS1) abolished virus replication *in vitro* (Fan *et al.*, 2014).

In regard of AcMNPV LEF-2, it would be also of interest to determine how the other identified conserved amino acids (Tyr24, Phe43, Gly47, Leu57, Arg130, Tyr159, Lys164) may be required for protein function (Table 4.2).

The analysis of the *lef-2* transcription profiles in virus-infected *T. ni* cells revealed that the virus MOI, the cell line and the virus strain may impact its expression (Fig. 4.20). While transcription of *lef-2* was highest in AcMNPV C6-infected *T. ni* Hi5 cells (MOI 5) at 12 h p.i., expression in AcMNPV E2-infected Tnms42 cells peaked at 36 h p.i. indicating a role for LEF-2 in virus replication and an additional one at later time points during the viral replication cycle. This idea is further supported by the two *lef-2* promoter motifs generating

an early and a late transcript (Ahrens and Rohrmann, 1995; Sriram and Gopinathan, 1998; Chen *et al.*, 2013).

To investigate the translation and localisation profiles of AcMNPV LEF-2 and how the mutagenesis of the protein disrupt both profiles, an antibody directed against the LEF-2 N-terminus was generated (GeneScript®). Unfortunately, the generated antibody detected a protein with the wrong molecular mass (~35kDa) in virus-infected cell lysates after western blotting (data not shown). The AcMNPV LEF-2 protein has a predicted molecular weight of 23.9 kDa (Ayres *et al.*, 1994) and a c-MYC-tagged LEF-2 was running at a size of ~25 kDa on protein gels as detected after immunoblotting using a c-MYC specific antibody of virus-infected cell lysates (R. D. Possee, personal communication). As 5' *c-myc*-tagging but not 3' of AcMNPV *lef-2* was previously successful, attempts were made to recreate this virus (Fig. 4.21). However, after plaque purification of co-transfected cells with linearised AcΔ*lef-2* DNA with the appropriate transfer vector, the *c-myc* tag of the generated was specifically lost. Even the exclusion of a titration resulted in the loss of the *lef-2 c-myc* tag. Only a co-transfection with circular AcΔ*lef-2* DNA preserved the *lef-2* tag (data not shown). The recombinant Ac.mycLEF-2 generated BV titre was not different to wt-infected cells (Fig. 4.19D) but intracellular viral DNA levels were dramatically reduced (Fig. 4.18) suggesting a mixed Ac.mycLEF-2/AcΔ*lef-2* population. Overall, this indicated that the LEF-2 N-terminus is also crucial for protein function and any modification resulted in the need of a helper virus required for virus replication.

Unfortunately, test expression experiments of Ac.mycLEF-2 infected *Sf9* cells revealed that c-MYC could not be detected after running virus-infected cell lysates on a protein gel and a subsequent western blotting and immunostaining against c-MYC. A positive control expressed a c-MYC-tagged protein (data not known). However, there were differences in the protocol how the Ac.mycLEF-2 was generated to the previous protocol. Although the *c-myc-lef-2* nucleotide sequence was the same in both approaches, the backbone used for virus generation varied. While in the recent approach the backbone virus AcΔ*lef-2* DNA was generated in a bacmid system, the AcΔ*lef-2*^{sup4-O⁹} DNA was produced in a yeast system (R. D. Possee, personal communication). It could be speculated that the difference in the system may account for the previous success of c-MYC-LEF-2 expression in virus-infected cells.

4.8 Conclusion

The aim of this chapter was to examine the role of AcMNPV LEF-2 in viral replication and viral very late gene expression by mutagenesis studies (Fig. 4.22; Table 4.4). While the role of *lef-2* in very late gene expression could not be completely determined, it may be hypothesised that it has an additive effect by acting together with *pk-1*. However, different

LEF-2 domains such as the highly conserved cysteine residues and the highly conserved alanine 154 residues are required for effective viral DNA replication. The initial motivation for this work was to follow up a previous study (Merrington *et al.*, 1996), which reported the original VLD1 mutant. The experiments described here highlight the difficulties of working with a virus mutant derived using chemical mutagenesis during virus replication. Multiple gene mutations are possible. Given the ease with which synthetic genes and viruses may now be produced, technology that was not available 20 years' ago, these represent vastly improved approaches to the study of virus gene function.

Chapter Five:

RNA-sequencing analysis of the effects of a *vp39* knock- down by RNAi

This chapter was carried out in Garry W. Blissard's lab at Boyce Thompson Institute (BTI) in the USA under a visiting scholarship program for 4.5 month in summer 2017; funded in part by Santander Scholarship. Virus infections were performed by G. Ya (BTI) and bioinformatics analyses (data cleaning and trimming, alignment to reference genome and calculation of RPKM values) were carried out by K. Bao (BTI). I generated the strand-specific RNA-sequencing libraries and performed all downstream analyses at BTI.

5.1 Introduction

The viral structural protein VP39 is the major component of the viral capsid in both BVs and ODVs. Moreover, it is one of the three most abundant viral proteins in BVs (Wang *et al.*, 2010b). Deletion of the *vp39* coding region abolished progeny virion production in virus-infected cells (Ono *et al.*, 2012).

The major capsid protein is expressed as three late mRNAs, which are maximally transcribed between 12-24 h p.i. in virus-infected cells (Thiem and Miller, 1989b). Transcription is initiated at nucleotide (nt) positions -58, -106 and -322 relative to the *vp39* ATG motif. The PAS is located at position +163 nt relative to the *vp39* stop codon (Chen *et al.*, 2013).

Although VP39 has been mainly recognised as a viral structural protein, mutagenesis of the highly conserved glycine 276 of VP39 was implicated to play a crucial role in *p10* and *polh* expression in BmNPV infected cells as examined by qPCR (Katsuma and Kokusho, 2017). Moreover, the knock-down of *vp39* by RNAi in AcMNPV-infected cells resulted in a decreased accumulation of viral P10 on a polyacrylamide gel compared to a control knock-down (*lacZ*; G. Ya, G. W. Blissard, personal communication). This made *vp39* a prime candidate gene required for the transition from viral late to very late gene expression in baculovirus-infected cells. Hence, the effects of a *vp39* knock-down by dsRNAs on the viral transcriptome and the viral very late genes were investigated in more detail by strand-specific sequencing (RNA-seq; Fig. 5.1A).

Whole-transcriptome analyses and gene expression studies by Next Generation Sequencing (NGS) produce an enormous volume of data by massive parallel high-throughput sequencing. It is also referred to as RNA-seq and produces a snapshot of the transcriptome. Unique combinations of protocols exist for specific platforms, which include template (library) generation, sequencing and data analysis. One of the most frequently utilised platforms is from Illumina® (reviewed by Marioni *et al.*, 2008; Wang *et al.*, 2009; Metzker, 2010; Ozsolak and Milos, 2011; Sharma and Vogel, 2014; Conesa *et al.*, 2016).

A current method (Fig. 5.1B) for the generation of libraries for RNA-seq involves the isolation of polyadenylated (polyA) RNA followed by their random fragmentation. The fragments are ultimately used as templates for clonal amplification by PCR, for which adapters were ligated to the target ends. Adapters contain the universal priming sites. Moreover, during PCR enrichment, libraries are tagged by the addition of six unique nucleotides (indices) allowing the pooling (by multiplexing) for simultaneous sequencing of a high number of libraries at once. Indices can be identified and sorted at the beginning of the data analysis. For the preservation of strand information, strand-specific RNA-seq can

be performed by the selective chemical marking of the non-coding strand by uracil (Fig. 5.1B; Craig *et al.*, 2008; Parkhomchuk *et al.*, 2009; Quail *et al.*, 2009; Zhong *et al.*, 2011).

Before the actual sequencing process begins (Fig. 5.1C), libraries are loaded onto the Illumina® flow cell, where they hybridise on a lawn of surface-bound oligos, which are complementary to the library adapter sequences. Clonal clusters are generated by bridge amplification (Illumina Sequencing Manual).

Base-by-base sequencing occurs by the cyclic reversible terminator-based method, of which the principle “sequencing-by-synthesis” relies on Sanger sequencing. During each sequencing cycle, all fluorescently labelled nucleotides are present and the DNA polymerase incorporates the fluorescently labelled nucleotide into the growing strand, which is complementary to the template base. Imaging is performed to identify the nature of the nucleotide, after which the inhibiting group of the fluorescent dye is cleaved off and a new sequencing cycle begins (reviewed by Metzker, 2010; Chen, 2014). Sequencing can be either performed from one end (single end-SE) or from both sides (paired end-PE), whereby the latter is preferred for the initial (*de novo*) transcriptome assembly (Ni *et al.*, 2010). Moreover, longer reads help for read mapping, transcriptome assembly and gene expression analysis although shorter reads are adequate for well-annotated organisms. The same holds true for the number of reads that are required for data analysis. As a rule of thumb ~10 million reads per library are sufficient to accurately determine gene expression and to perform further downstream analysis. However, the larger the genome size, the more reads should be obtained (Garber *et al.*, 2011; Tarazona *et al.*, 2011; Vijay *et al.*, 2013).

Data analysis usually includes the de-multiplexing of templates, the removal of adapter and rRNA sequences and low quality reads from raw reads (Bolger *et al.*, 2014). Final cleaned reads are either mapped to a reference transcriptome or genome or the transcriptome is assembled *de novo*. Typically, differential gene expression is analysed afterwards (Fig. 5.1D; reviewed by Marioni *et al.*, 2008; Wang *et al.*, 2009; Metzker, 2010; Ozsolak and Milos, 2011; Sharma and Vogel, 2014; Conesa *et al.*, 2016).

The hypothesis that VP39 is involved in very late gene expression is a significant contrast with its current defined role as a virus structural protein, which is beyond doubt. The methods utilised in this study also offer a very different approach to those used in Chapter 4 to study LEF-2 function.

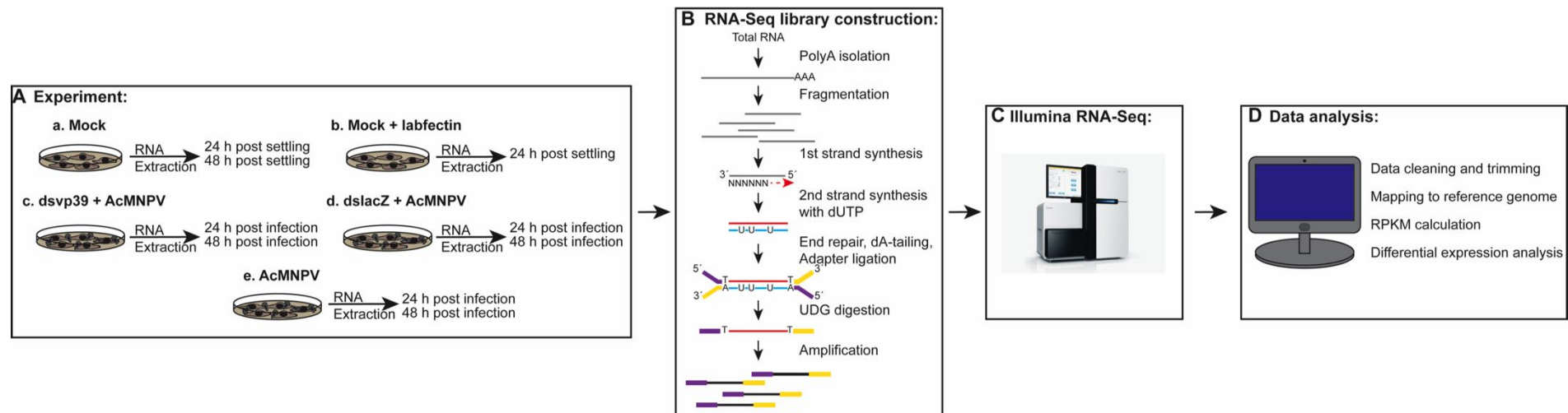


Figure 5.1 vp39 RNAi and RNA-seq experimental design

A) Total RNA of a.) Tnms42 cells b.) labfectin-treated Tnms42 cells or c.) dsvp39 RNA or d.) dsIacZ RNA-transfected and subsequently AcMNPV-infected Tnms42 cells or e.) AcMNPV-infected Tnms42 was extracted at indicated time points. B) Strand-specific libraries for RNA-seq were generated as previously described (Zhong *et al.*, 2011). C) Sequencing was outsourced (Illumina®, Weill Cornell Medical School). D) Raw reads were cleaned and trimmed and mapped against the AcMNPV and reads per kilobase per million base mapped reads (RPKM) values were calculated.

5.2 Knock-down of *vp39* by RNAi and strand-specific library generation for RNA-sequencing

To determine the effects of a *vp39* knock-down by RNAi, 2×10^5 Tnms42 cells (in a 24-well format) were transfected with 5 μ g dsRNA either against *vp39* (samples referred to as “dsvp39”) or alternatively against *lacZ* (samples referred to as “dslacZ”) for a control knock-down and subsequently infected with AcMNPV E2 MOI 10 (G. Ya; Fig. 5.1A, 5.2). In this chapter AcMNPV E2 is referred to as wt. The AcMNPV E2 is a different isolate than AcMNPV C6 (Smith and Summers, 1979). As a further control Tnms42 cells were infected with AcMNPV (MOI 10; samples referred to as “no dsRNA”; G. Ya). The Tnms42 cell line is a clonal derivative of the *T. ni* Hi5 cell. In contrast to *T. ni* Hi5 cells, which are persistently infected with an alphavirus designated as Tn5 cell line virus (TNCL), the Tnms42 cells contain no detectable alphavirus (Li *et al.*, 2007; Chen *et al.*, 2013). Virus-infected cells were harvested either at 24 or 48 h p.i. (G. Ya). As further controls Tnms42 cells were mock-transfected with lipofectin (samples referred to as “mock labfectin”; G. Ya; Campbell, 1995) or not treated at all (samples referred to as “mock untreated”) and harvested either 24 or 48 h post-seeding (Fig. 5.1A). Total RNA was extracted using TRIzol (Invitrogen™) and RNA degradation and contamination was examined using 1% (w/v) agarose gels stained with ethidium bromide (data not shown).

It was decided to analyse samples by strand-specific RNA-seq to preserve the information of which DNA strand was the original template during mRNA transcription. Moreover, the Illumina® platform HiSeq4000 was utilised with 50 bp SE reads. The rather short SE reads were sufficient for gene expression studies in the well-annotated AcMNPV, which in addition contains a small genome of ~140 kbp encoding for about 156 orfs (reviewed by Conesa *et al.*, 2016).

Strand-specific RNA-seq libraries were generated as previously described (3 μ g of total RNA was utilised; Fig. 5.1B; Zhong *et al.*, 2011), multiplexed and pooled as indicated in Table 5.1. In total 19 libraries were pooled per lane. Taking into account that Illumina® HiSeq4000 generates 380-400 million reads per lane, an average number of ~20 million reads per library was expected. The expected number of reads would be acceptable for mapping not only against AcMNPV but also against the *T. ni* genome if required (Chen *et al.*, 2013, 2014).

Before Illumina® RNA-seq was outsourced to Epigenomic core (Weill Cornell Medical School, NYC, US; Fig. 5.1C), the quality of both pooled libraries was investigated by running the pre-purified libraries on a 1% (w/v) gel electrophoresis (Fig. 5.3). The mean of DNA fragments in each of the pooled library was less than 300 bp with the largest DNA

fragments just bigger than 500 bp. The optimal size of DNA fragments is determined by the limitations of the NGS platform, which for Illumina® instruments is in general <500 bp with a peak of ~250-300 bp (Illumina Sequencing Manual).

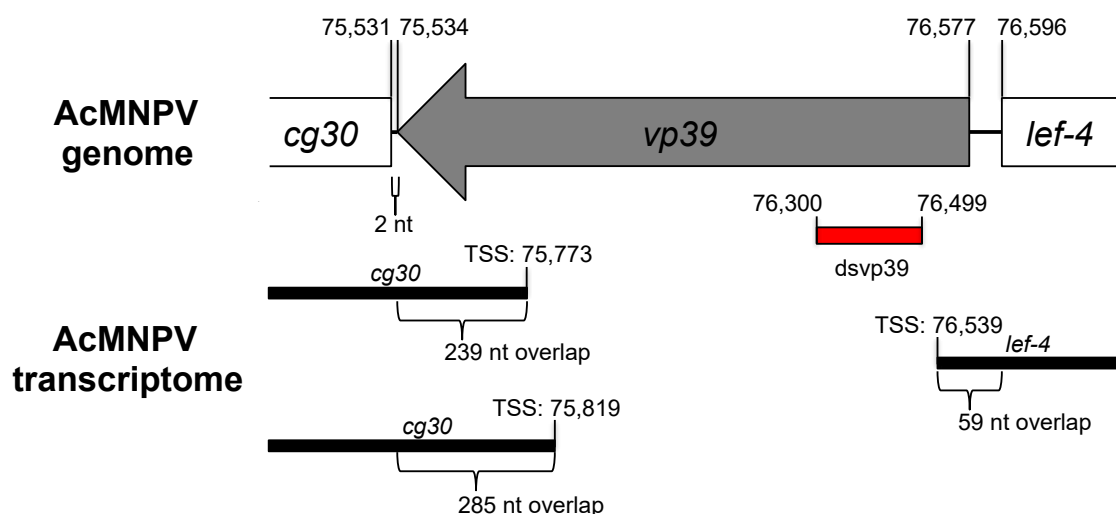


Figure 5.2 AcMNPV *vp39* genomic and transcriptomic organisation

Shown is the AcMNPV *vp39* genome and transcriptome. Orfs are shown as arrows, which indicate direction of transcription. Black boxes indicate transcripts and red box shows nucleotide (nt) sequence against which the *vp39* dsRNA is located. Numbers above arrows and boxes indicate AcMNPV genome position. Downstream of the 1043 bp *vp39* orf (grey) in antiparallel direction located is the *lef-4* orf and upstream with only 2 nt in between located is the *cg30* orf. *Cg30* is transcribed in the same direction as *vp39* and transcription occurs from an early (TSS: 75,773) and late (TSS: 75,819) promoter. *Lef-4* is transcribed from an early promoter in antisense direction (TSS: 76,539). Both the *lef-4* and the *cg30* transcripts overlap with *vp39*. However, the *dsvp39* RNA (red) was designed to only target *vp39* transcripts (76,300-76,499). TSS: Transcriptional start site (TSS data taken from Chen *et al.*, 2013).

5.3 RNA-sequencing Data analysis

The generated RNA-seq reads were de-multiplexed, adapters, poly A/T (polyadenylated, adenine/thymine) and rRNA sequences were removed (K. Bao). Reads were mapped against tetravirus and alphanodavirus genomes to remove those sequences (K. Boa; Table 5.2). Mapping against tetra- and alphanodaviruses were performed as a quality control. Tetraviruses specifically infect lepidopteran species but can also replicate *in vitro* (Moore *et al.*, 1985; Walter *et al.*, 2010). As expected, no reads could be mapped to the tetravirus genome (Table 5.2). However, several thousand reads from AcMNPV-infected Tnms42 cells mapped to the alphanodavirus genome (Table 5.2), which represented ~0.1-0.3% of the total number of reads. As alphanodaviruses contain a positive-sense RNA genome the number of reads is not quantitative as their genome can function as mRNAs. Alphanodaviruses do not transcribe polyA RNA (Li *et al.*, 2007). On the contrary, no reads from mock-infected Tnms42 cells could be mapped to the alphanodavirus genome (Table 5.2). This indicated that either the inoculum used for the infection studies was contaminated with alphanodavirus, which could be tested by qPCR or alternatively,

that infection stimulated the transcription of alphanodavirus in AcMNPV-infected cells (Li *et al.*, 2007). Nevertheless, it was decided to continue with the analysis.

Table 5.1 *vp39* RNAi: Pooled libraries for Illumina® RNA-seq

Pooled libraries no 1*, final concentration 7.2 ng/μl in 21 μl TE buffer		
Sample	Illumina® index	Index sequence
Mock labfectin 24 h 1	1	CGTGAT
Mock labfectin 24 h 2	2	ACATCG
Mock labfectin 24 h 3	3	GCCTAA
Mock untreated 24 h 1	13	TTGACT
Mock untreated 24 h 2	14	GGAAC
Mock untreated 24 h 3	15	TGACAT
Mock untreated 48 h 1	16	GGACGG
Mock untreated 48 h 2	17	CTCTAC
Pooled libraries no 2**, final concentration 10.5 ng/μl in 21 μl TE buffer		
Sample	Illumina® index	Index sequence
Mock untreated 48 h 3	18	GCGGAC
dslacZ 24 h p.i. 1	21	CGAAAC
dslacZ 24 h p.i. 2	4	TGGTCA
dslacZ 24 h p.i. 3	5	CACTGT
dslacZ 48 h p.i. 1	19	TTTCAC
dslacZ 48 h p.i. 2	25	ATCAGT
dslacZ 48 h p.i. 3	28	CTTTTG
dsvp39 24 h p.i. 1	30	CCGGTG
dsvp39 24 h p.i. 2	22	CGTACG
dsvp39 24 h p.i. 3	23	CCACTC
dsvp39 48 h p.i. 1	24	GCTACC
dsvp39 48 h p.i. 2	37	ATTCCG
dsvp39 48 h p.i. 3	40	TCTGAG
no dsRNA 24 h p.i. 1	45	GAATGA
no dsRNA 24 h p.i. 2	9	CTGATC
no dsRNA 24 h p.i. 3	10	AAGCTA
no dsRNA 48 h p.i. 1	11	GTAGCC
no dsRNA 48 h p.i. 2	20	GGCCAC
no dsRNA 48 h p.i. 3	29	TAGTTG

*Illumina RNA-Seq lane 1 contained pooled samples no 1 that was shared with a different project (A. Schrestha, BTI); In total, lane no 1 contained 19 pooled libraries with a final concentration of 7.2 ng/μl

** Illumina RNA-Seq lane 2 contained pooled samples no 2 only and was not shared

Quality control of reads after their final clean revealed that more than 15 million reads per sample was generated except for sample “no dsRNA 48 h p.i. 2”, which only obtained about seven million reads. However, this was still sufficient for any downstream analysis and differential expression analysis of viral genes (Table 5.2; Vijay *et al.*, 2013; G. W. Blissard, personal communication). Filtered reads were mapped to the AcMNPV genome (Accession no. NC_001623; K. Bao; supplementary CD Table S 5.1; Ayres *et al.*, 1994; Chen *et al.*, 2013) and RPKM values of all viral genes were calculated (K. Bao).

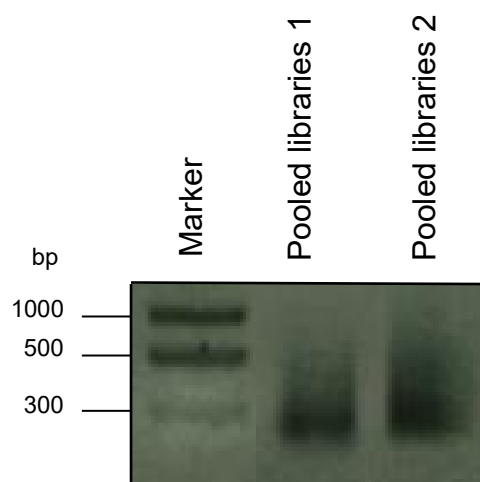


Figure 5.3 Agarose gel of pooled RNA-seq libraries

To analyse the length distribution of the DNA fragments from the pooled libraries, 2 μ l of each purified pooled library was run on a 1% (w/v) agarose gel and stained with ethidium bromide.

To minimise the effects of overlapping transcripts resulting from internal transcription initiation in upstream orfs and transcripts that overlap with downstream orfs, the calculation of AcMNPV gene expression values (RPKM) were adjusted as previously reported (Chen *et al.*, 2013) and as indicated in Table S 5.2 (supplementary CD). Typically, the non-overlapping regions of orfs with overlapping 5' or 3' transcripts from adjacent genes were used for viral gene expression calculation and analysis. However, transcripts for some viral orfs share co-terminal 3' PAS and have distinct 5' TSS. In those cases, the RPKM value determined for the downstream orf was adjusted by subtracting the RPKM value of the upstream viral orf (Chen *et al.*, 2013). However, in some cases the subtraction led to negative RPKM values. This occurred especially when the upstream viral gene was expressed very strongly. As gene expression cannot be negative, those values were considered as 0 for any further downstream analysis of viral gene expression.

5.4 Effects of *vp39* RNAi on the viral transcriptome

For the analysis of the effect of a *vp39* knock-down on viral gene expression, the fold change (FC) of gene expression as a $\log_2$ ratio of RPKM values between the following two samples sets were calculated: 1) The $FC_{\log_2}$ of samples of the control (dslacZ) and the *vp39* knock-down (dsvp39); 2) The $FC_{\log_2}$ between the control knock-down (dslacZ) and samples that were infected only (no dsRNA) to investigate the effect of the transfection of an unspecific dsRNA on transcription. Viral genes with $FC_{\log_2} > 0.5$ (FC ~ 1.41) or $FC_{\log_2} < -0.5$ (FC ~ 0.71 ; with $p < 0.05$, Student's t-test) for at least one time point p.i. were considered as up or down regulated (Fig. 5.4). A rather low $FC_{\log_2}$ was selected as a cut off to identify any changes in viral gene expression.

Table 5.2 Illumina® RNA-seq data cleaning summary

sample	total raw reads	remove adapter, low quality		remove polyA/T		remove rRNA		clean	remove Tetravirus		remove Alphanodovirus		final clean
		clean	%of raw	No. polyA/T	%polyA/T	No. reads	%rRNA		No. Tet	%Tet	No. Alph	%Alph	
Mock untreated 24 h 1	30,708,435	29,948,459	97.53	14,549	0.05	111,794	0.37	29,822,116	0	0.00	0	0.00	29,822,116.00
Mock untreated 24 h 2	32,821,139	32,145,774	97.94	18,351	0.06	126,031	0.39	32,001,392	0	0.00	0	0.00	32,001,392.00
Mock untreated 24h 3	30,975,060	29,810,537	96.24	44,650	0.15	109,523	0.37	29,656,364	0	0.00	0	0.00	29,656,364.00
Mock untreated 48 h 1	34,623,720	33,954,533	98.07	10,719	0.03	80,051	0.24	33,863,763	1	0.00	0	0.00	33,863,762.00
Mock untreated 48 h 2	33,825,024	33,010,002	97.59	52,642	0.16	67,157	0.20	32,890,203	0	0.00	0	0.00	32,890,203.00
Mock untreated 48h 3	17,087,148	16,621,306	97.27	37,114	0.22	44,411	0.27	16,539,781	0	0.00	77	0.00	16,539,704.00
Mock labfectin 24h 1	29,414,221	28,981,583	98.53	11,166	0.04	124,868	0.43	28,845,549	0	0.00	0	0.00	28,845,549.00
Mock labfectin 24h 2	33,372,312	32,889,250	98.55	14,452	0.04	196,902	0.60	32,677,896	0	0.00	0	0.00	32,677,896.00
Mock labfectin 24h 3	28,988,345	28,580,048	98.59	5,867	0.02	161,188	0.56	28,412,993	0	0.00	0	0.00	28,412,993.00
dslacZ 24 h p.i. 1	17,011,653	16,600,841	97.59	30,908	0.19	156,316	0.94	16,413,617	0	0.00	17,559	0.11	16,396,058.00
dslacZ 24 h p.i. 2	15,528,045	15,110,744	97.31	32,168	0.21	109,032	0.72	14,969,544	0	0.00	22,539	0.15	14,947,005.00
dslacZ 24 h p.i. 3	16,945,183	16,473,209	97.21	24,019	0.15	58,007	0.35	16,391,183	0	0.00	20,514	0.13	16,370,669.00
dslacZ 48 h p.i. 1	19,389,750	18,917,708	97.57	16,336	0.09	193,494	1.02	18,707,878	0	0.00	30,573	0.16	18,677,305.00
dslacZ 48 h p.i. 2	21,094,832	20,590,983	97.61	19,015	0.09	760,764	3.70	19,811,204	0	0.00	38,525	0.19	19,772,679.00
dslacZ 48 h p.i. 3	17,141,817	16,555,357	96.58	70,261	0.42	72,698	0.44	16,412,398	0	0.00	33,525	0.20	16,378,873.00
dsvp39 24 h p.i. 1	20,031,483	19,469,053	97.19	21,417	0.11	243,712	1.25	19,203,924	0	0.00	36,913	0.19	19,167,011.00
dsvp39 24 h p.i. 2	21,301,695	20,538,387	96.42	12,320	0.06	75,071	0.37	20,450,996	0	0.00	41,264	0.20	20,409,732.00
dsvp39 24 h p.i. 3	19,945,214	19,511,254	97.82	6,968	0.04	181,705	0.93	19,322,581	0	0.00	26,326	0.14	19,296,255.00
dsvp39 48 h p.i. 1	18,317,249	17,908,065	97.77	6,928	0.04	178,730	1.00	17,722,407	0	0.00	24,154	0.14	17,698,253.00
dsvp39 48 h p.i. 2	24,709,133	23,996,229	97.11	64,793	0.27	153,508	0.64	23,777,928	0	0.00	72,455	0.30	23,705,473.00
dsvp39 48 h p.i. 3	18,440,693	17,988,482	97.55	16,521	0.09	510,558	2.84	17,461,403	0	0.00	33,569	0.19	17,427,834.00
no dsRNA 24 h p.i.1	16,679,898	16,219,688	97.24	66,121	0.41	56,660	0.35	16,096,907	0	0.00	33,281	0.21	16,063,626.00
no dsRNA 24 h p.i. 2	15,449,179	15,104,409	97.77	5,968	0.04	137,143	0.91	14,961,298	0	0.00	22,435	0.15	14,938,863.00
no dsRNA 24 h p.i. 3	25,008,153	24,307,526	97.20	16,541	0.07	112,006	0.46	24,178,979	0	0.00	30,330	0.13	24,148,649.00
no dsRNA 48 h p.i. 1	22,128,897	21,643,715	97.81	28,518	0.13	109,788	0.51	21,505,409	0	0.00	46,655	0.22	21,458,754.00
no dsRNA 48 h p.i. 2	14,498,533	9,879,219	68.14	2,841,542	28.76	8,254	0.12	7,029,423	0	0.00	2,881	0.04	7,026,542.00
no dsRNA 48 h p.i. 3	16,901,423	16,444,803	97.30	23,390	0.14	88,041	0.54	16,333,372	0	0.00	37,679	0.23	16,295,693.00

Abbreviations: Tet: tetravirus, Alph: alphanodavirus, polyA/T: polyadenylated, adenine/thymine

As a non-targeted knock-down (*dslacZ*) is considered as the best suitable control for a targeted knock-down (*dsvp39*) during RNAi (reviewed by Huppi *et al.*, 2005), it was decided not to compare the conditions “*dsvp39*” with no “dsRNA”.

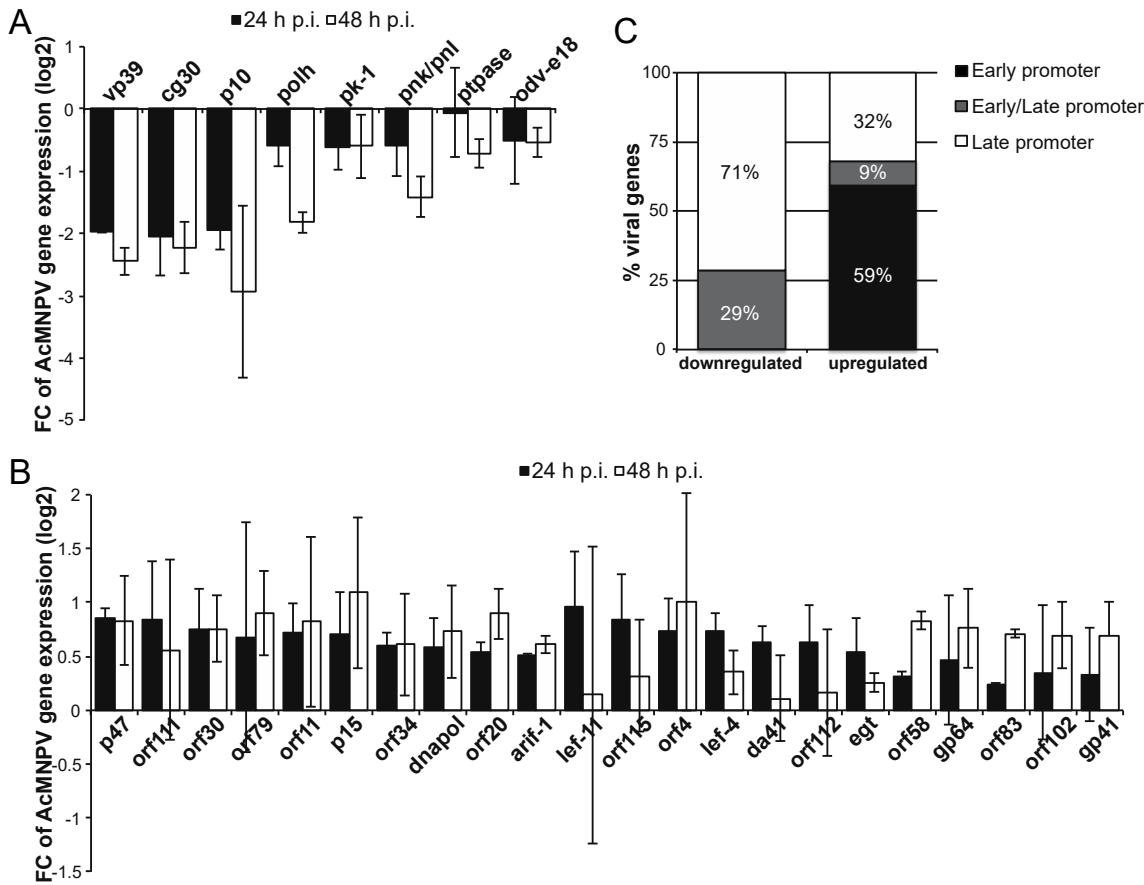


Figure 5.4 Effects of a *vp39* knock-down on a viral transcriptome

A,B) The FC_{log2} of viral gene expression in AcMNPV-infected Tnms42 cells (MOI 10) between the control (*dslacZ*) and *vp39* knock-down were calculated. An A) $FC_{log2} < -0.5$ and B) $FC_{log2} > 0.5$ for at least one time point p.i. (with $p < 0.05$, Student's t-test, compare Fig. 5.6, 5.7) were considered as up or down regulated. C) Promoter motifs of viral genes considered as down or up regulated after *vp39* RNAi (promoter motifs adapted from Chen *et al.*, 2013).

The knock-down of *vp39* resulting from the *vp39*-targeted dsRNA caused a reduction of its expression by 71% at 24 h.p.i and 81% at 48 h.p.i. (Fig. 5.5). Expression of *cg30*, and the very late genes *p10* and *polh* were the three most highly reduced genes in virus-infected cells besides *vp39*. Their transcription was reduced by 78%, 82% and 71%, respectively at 48 h.p.i. (Fig. 5.5).

Interestingly, *cg30* transcription was almost as highly down regulated as *vp39* (Fig. 5.4A, 5.5). The *cg30* orf is located 2 nt upstream of the *vp39* stop codon and the *cg30* transcript initiates within the *vp39* orf, approximately 26% (286 nt) from the 3' end of the *vp39* (Fig. 5.2; Chen *et al.*, 2013). However, the dsRNA against *vp39* targeted a nucleotide region downstream of the overlapping mRNAs, which should not affect the *cg30* transcript (Fig. 5.2). Additionally, the *lef-4* orf is located downstream of *vp39* orientated in the

opposite direction. The 5' end of the *lef-4* transcripts overlaps the *vp39* orf by 38 nt but the *vp39* dsRNA targeted nucleotides more upstream of this overlap and should thereby not affect *lef-4* expression either (Fig. 5.2). However, transcription of *lef-4* was up regulated after transfection with *vp39* dsRNAs when compared to the control knock-down (Fig. 5.4B, 5.7N).

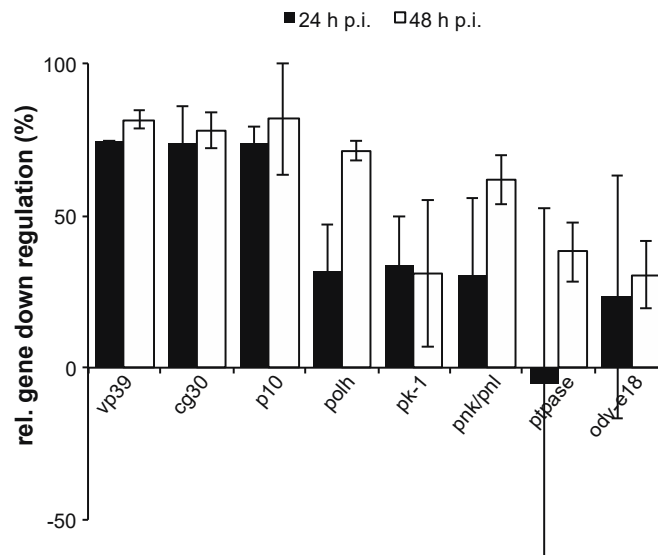


Figure 5.5 Relative down regulated viral gene caused by *vp39* RNAi

Down regulated viral genes after *vp39* RNAi and subsequent infection with AcMNPV E5 (MOI 10). Shown is the relative down regulation of viral gene expression (from Fig. 5.4A) compared to a control knock-down (*lacZ*).

In total, the expression of eight viral genes were considered as down regulated during a *vp39* knock-down when compared to a *lacZ* knock-down: *cg30*, *p10*, *polh*, *pk-1*, *ptpase*, *pnk/pnl*, *odv-e18* (Fig. 5.4A, 5.5, 5.6). Interestingly, it appeared that most (71%) of the down-regulated viral genes contained a late promoter motif (Fig. 5.4C; Chen *et al.*, 2013).

On the contrary to the down regulated viral genes after *vp39* RNAi, transcription of a number of genes were significantly up regulated when compared to the *lacZ* control knock-down (Fig. 5.4B, 5.7). These viral genes include: *p47*, *orf11*, *orf30*, *orf79*, *orf11*, *p15*, *orf34*, *dnapol*, *orf-20*, *arif-1*, *lef-11*, *orf115*, *orf4*, *lef-4*, *da41*, *orf112*, *egt*, *orf58*, *gp64*, *orf83*, *orf102* and *gp41*. Most of these up regulated viral genes (59%) contained an early promoter motif (Fig. 5.4C; Chen *et al.*, 2013).

5.5 The effects of a non-specific knock-down by RNAi on the viral transcriptome

To analyse the effect of a transfection of (non-specific) dsRNAs on viral gene expression, transcription of virus-infected cell either transfected with the control *lacZ* dsRNA or with no

dsRNA were compared and as before the FC of AcMNPV gene expression was calculated as a $\log_2$ difference. In general, no viral genes were significantly up regulated at 24 h p.i. or 48 h p.i. and moreover, no viral genes were significantly down regulated at 48 h p.i. However, the expression of three viral genes were considered as reduced at 24 h p.i., which were: *orf107*, *dbp* and *egt* (Fig. 5.8).

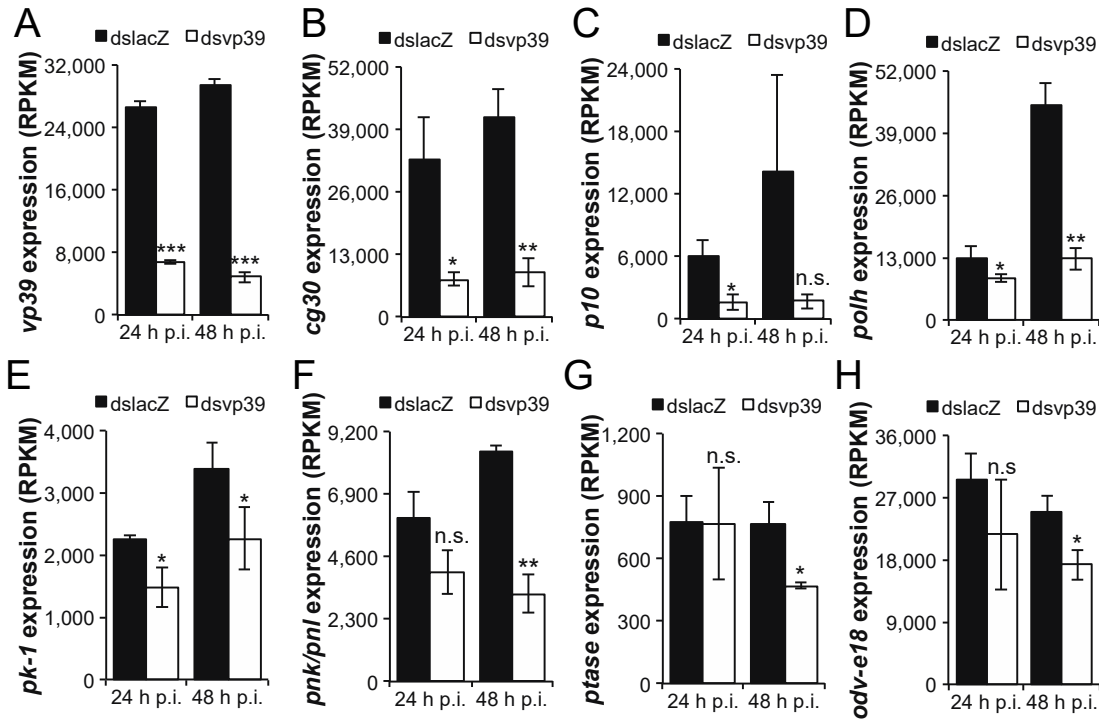


Figure 5.6 Down regulated viral gene by *vp39* RNAi

Down regulated viral genes after *dsvp39* RNA knock-down and subsequent infection with AcMNPV (MOI 10). Shown are only viral genes that were significantly down regulated after *vp39* RNAi compared to a control knock-down (*lacZ*) with a fold change (FC on a $\log_2$ scale) of viral gene expression of at least -0.5 (compare Fig. 5.4A and Fig. 5.5). Significance was calculated by Student's t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: not significant

5.6 Discussion

The viral protein VP39 is the major capsid protein in both BVs and ODVs and is one of the three most abundant proteins in BV. So far, VP39 has been only associated with structural functions (Pearson *et al.*, 1988; Thiem and Miller, 1989b).

The knock-down of *vp39* in virus-infected cells affected a surprisingly low number of viral genes when compared to a control RNAi (Fig. 5.4). Twenty-nine viral genes were differentially expressed at least at one time point p.i., whereby the transcription of seven genes (without *vp39*) were down regulated and 22 genes were up regulated. When the delayed early gene *pp31* (39K) was knocked out and viral gene expression was examined by an oligonucleotide microarray, the expression of 60 genes was decreased to 30-50% to

that of the repair virus. Expression of six genes (*p6.9*, *orf97*, *orf60*, *orf98*, *orf102*, *chitA*) was more dramatically reduced to 20-33% of the level of the repair virus (Yamagishi *et al.*, 2007). Moreover, when the immediate early transactivator *pe38* was knocked out the global viral gene expression altered early during the viral replication cycle (3 h p.i.) but expression returned to the same level as in wt-infected cell later (72 h p.i.) as examined by a microarray indicating that other viral genes may compensate for the loss of *pe38* (Jiang *et al.*, 2006).

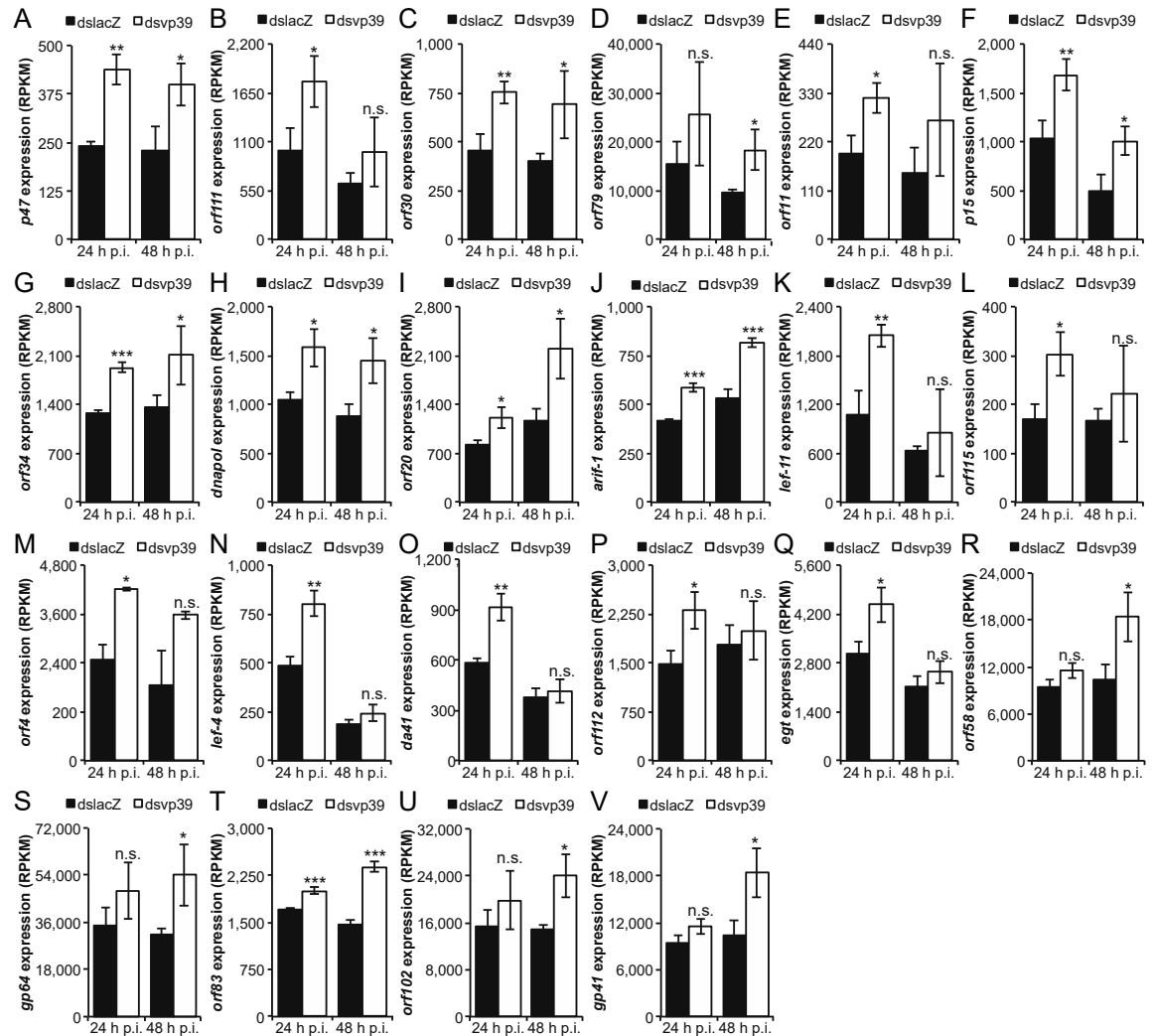


Figure 5.7 Up regulated viral gene by *vp39* RNAi

Up regulated viral genes after *dsvp39* RNA knock-down and subsequent infection with AcMNPV (MOI 10). Shown are only viral genes that were significantly up regulated after *vp39* RNAi compared to a control knock-down (*lacZ*) with a fold change (FC on a log₂ scale) of viral gene expression of more than 0.5 for at least one time point p.i. (compare Fig. 5.4B). Significance was calculated by a Student's t-test: **p*<0.05, ***p*<0.01, ****p*<0.001, n.s.: not significant

The knock-down of *vp39* by RNAi and the subsequent analysis of the viral transcriptome by RNA-seq revealed that the knock-down also affected the expression of other viral genes (Fig. 5.4A, B). Particularly interesting was the effect on viral very late gene expression and the down regulation of *polh* and *p10* expression (Fig. 5.6C, D). Both *p10*

and *polh* are hyperexpressed at the end of the viral replication cycle and are nonessential for viral replication. Therefore, they have been adapted to express recombinant proteins (Introduction 1.5.6, 1.7; Smith *et al.*, 1983c, I 1983b; Pennock *et al.*, 1984; Knebel *et al.*, 1985). That VP39 may have an additional function, other than just being the main capsid protein, by regulating viral very late gene expression was already hypothesised before (Katsuma and Kokusho, 2017). Infection of BmN-4 cells with a recombinant BmNPV, in which the highly conserved glycine 276 in VP39 was substituted with serine, reduced both *p10* and *polh* expression, whereas *vp39* transcription was not affected when compared to wt-infected cells as examined by RT-PCR. The reduction of very late genes in turn decreased OB production. At the same time, expression of the two viral genes *vlf-1* and *fp25K* that are known to be required for very late gene expression was unaffected. The BVs generated by this mutant contained a smaller amount of both VP39 and DNA per virion but the level of GP64 was increased (Katsuma and Kokusho, 2017)

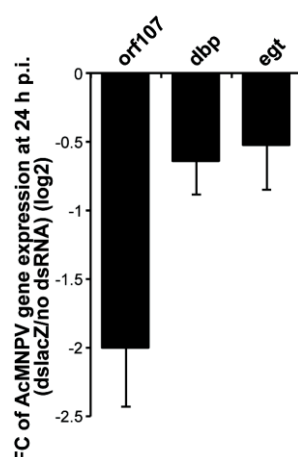


Figure 5.8 Differential expressed viral genes after non-specific (*lacZ*) dsRNA transfection

Differentially expressed viral genes at 24 h p.i. after *dslacZ* RNA knock-down and subsequent infection with AcMNPV (MOI 10). Shown are only viral genes that were significantly down regulated after *lacZ* RNAi compared to no knock-down (*lacZ*) with a fold change (FC on a log₂ scale) of viral gene expression of at least -0.5 (compare Fig. 5.4A and Fig. 5.5). Significance was calculated by a Student's t-test with $p < 0.05$. At 48 h p.i. viral genes were not significantly differentially expressed and no viral genes were significantly up regulated at 24 h p.i.

Moreover, expression of the viral kinase PK-1 was reduced after *vp39* RNAi (Fig. 5.6E). The PK-1 is a serine/threonine kinase lacking the N-terminal regulating domain that is usually found in protein kinases. It is expressed from 12 h p.i and accumulated to high levels very late in expression (Reilly and Guarino, 1994). This kinase was first identified as required for very late gene expression, from a ts mutant that expressed lower levels of very late RNA at the permissive temperature in infected cells (Fan *et al.*, 1996). In addition, cells infected with a *pk-1* knock-out virus did not produce infectious progeny. Electron microscopy studies revealed that the formation of nucleocapsids was affected and only masses of electron-lucent tubular structures were present in the nucleus when

cells were infected with this mutant virus. However, viral DNA replication did not seem to be affected (Liang *et al.*, 2013). The PK-1 appeared to phosphorylate either viral or host proteins that are essential for DNA packaging and nucleocapsid assembly (Liang *et al.*, 2013). In addition, data suggested that PK-1 may be part of the transcription initiator complex (TIC) that binds to the *polh* transcription initiation sequence, possibly regulating *polh* transcription (Mishra *et al.*, 2008). Therefore, *pk-1* may link *vp39* and viral very late gene expression. One could further speculate that PK-1 may be regulated by VP39 through a feedback loop.

It was also observed that the expression of the viral tyrosine/threonine phosphatase PTPase was reduced (Fig. 5.6G). The viral *ptpase* encodes for a tyrosine/serine phosphatase that accumulated to maximal transcript levels at 30 h p.i. (Kim and Weaver, 1993; Sheng and Charbonneau, 1993). The knock-out of the *ptpase* catalytic domain (vPTPdel) resulted in decreased BV production and partially defective ODV production in *Sf21* but not TN-368-infected cells. Pulse labelling with [³⁵S] methionine and [³⁵S] cysteine demonstrated no apparent differences in the protein synthesis kinetics between vPTPdel-infected *Sf21* and TN-368 cells compared to wt-infected cells. By pulse labelling of virus-infected *Sf21* cells with [³³P] orthophosphate, an additional 35 kDa phosphoprotein was detected at 24 h p.i., which could be encoded by either the viral or host genome. However, the same deletion mutant had no apparent effect on virus infectivity, virulence, or replication in *S. frugiperda* larvae (Li and Miller, 1995b). Most interestingly, the 19 kDa protein PTPase was associated with fibrillar structures in both the cytoplasm and nucleus of infected cell from 12 h p.i. throughout 48 h p.i. (Li and Miller, 1995a). These fibrillar structures may represent P10 and hence, VP39 may regulate viral very late gene expression directly or indirectly via PTPase through feedback loops.

Interestingly, the transcription of *cg30*, which is immediately downstream of *vp39*, is almost as highly reduced as *vp39* expression after *vp39* RNAi (Fig. 5.6B). The viral *cg30* is present in most alphabaculoviruses (~80%) and 91.3% of the *cg30* nucleotide sequence between AcMNPV and BmNPV is identical. The AcMNPV *cg30* encodes for a 30 kDa protein with two different DNA-binding motifs, a zinc finger and a leucine zipper motif. It was transcribed as early as 2 h p.i. and was present until 6 h p.i. and therefore, *cg30* is considered as an early gene. At later times p.i., *cg30* was described as a bicistronic transcript in a very low abundance that was initiated from the *vp39* late promoter and continued through *cg30* terminating at the same site as the early transcript (Thiem and Miller, 1989a). The existence of a bicistronic mRNA would at least partially explain the effect of a *vp39* knock-down on *cg30* transcription. In the same context, it was speculated that *cg30* (by acting as a transcriptional regulator) might be a prime candidate in the regulation of hyperexpressed genes. Moreover, a frameshift mutation during the

translation of the VP39 C-terminus would result in a VP39-CG30 fusion protein that would be capable of anchoring the viral genome via the CG30 DNA-binding motifs inside the capsid. However, translation of the bicistronic *vp39-cg30* transcript could never be verified (Thiem and Miller, 1989a).

In the genome of AcMNPV *cg30* seemed to be nonessential during the viral replication cycle. Insect cell lines (*Sf* or *T. ni*) or *T. ni* larvae that were infected with either a *cg30* deletion or *lacZ* replacement mutant produced both BV and ODV at wt levels. Protein synthesis kinetics analysed by pulse labelling with [³⁵S] methionine was similar in both wt and *cg30* mutant infected cells. However, *polh* levels were slightly decreased at 18 and 24 h p.i. (Passarelli and Miller, 1994b). On the contrary, mutations of *cg30* in BmNPV showed a more dramatic phenotype. Infection with a *cg30* knock-out mutant of both Bm5 cells and *B. mori* larvae resulted in lower BV titres, which could be rescued by a repair virus (Zhang *et al.*, 2012). Additionally, *cg30-lacZ* mutants produced fewer BVs *in vitro* and *in vivo*, whereas the OB production was only decreased *in vivo* and was not impaired *in vitro*. Interestingly, *polh* expression was reduced and *vp39* expression was slightly decreased in Bm5 cells as determined by qRT-PCR indicating that *cg30*, *vp39* and very late gene expression may get regulated through each other (Ishihara *et al.*, 2013).

Overall, if the effect on viral very late gene expression is caused by a *vp39* knock-down or if it is a combination of a reduced expression of both *vp39* and *cg30* this still needs to be determined. However, the generation of a *vp39* knock-out AcMNPV might be difficult as of massively overlapping transcripts. The alternative might be to introduce pre-mature stop codons into VP39. The knock-out of *cg30* alone did not seem to have dramatic effects on the AcMNPV replication cycle (Passarelli and Miller, 1994b).

Another down regulated viral gene after *vp39* RNAi-treatment was *pnk/pnl* (Fig.5.6F). It encodes a protein with RNA ligase, polynucleotide 5'kinase and polynucleotide 3'-phosphatase activity and it was therefore suggested that it might be part of a RNA repair pathway (Martins and Shuman, 2004). Infection of an insect cell line with a recombinant AcMNPV, in which *pnk/pnl* was replaced with *lacZ* demonstrated that BV production was unaffected when compared to AcMNPV-infected cells. Comparison of expression levels of several viral genes (GP64, IE1, VP39 and polyhedrin) in cells infected with the virus containing the *pnk/pnl* deletion showed no apparent differences by immunoblotting. In addition, pulse labelling of infected cells with [³⁵S] methionine and [³⁵S] cysteine demonstrated similar protein synthesis kinetics. Interestingly, the genomic region of *pnk/pnl* in AcMNPV 1.2 (a different virus strain than AcMNPV E2 and C6), GmMNPV and BmNPV was deleted as analysed by PCR and restriction enzyme digestion. Therefore, it was suggested that *pnk/pnl* is nonessential for virus survival (Durantel *et al.*, 1998).

However, *vp39* RNAi only moderately affected *pnk/pnl* transcription at 48 h p.i. ($FC_{\log 2} = -1.4$) compared to e.g. *p10* expression ($FC_{\log 2} \sim -3$) and had no significant effect at 24 h p.i. (Fig. 5.6F). Therefore, if and how VP39 may act together with PNK/PNL needs to be analysed in more detail.

The last viral gene that was down regulated as a result of *vp39* dsRNA was *odv-e18* (Fig. 5.6H). The *odv-e18* is transcribed from 16 h p.i. through 72 h p.i. Its gene product was characterised as a structural protein of the ODV envelope with a size of 18 kDa (Braunagel *et al.*, 1996, 2003). However, more recent mass-spec analyses indicated that ODV-E18 is also a component of AcMNPV BVs (Wang *et al.*, 2010b). It could be speculated if the major BV structural protein might be required for the expression of other structural viral proteins.

However, *vp39* knock-down did not only down regulate viral gene expression in virus-infected cells. In addition, a few viral genes were up regulated (Fig. 5.4B, 5.7). Most of the up regulated viral genes (59%; Fig. 5.4C) have in common that they are characterised as early genes indicating that the knock-down of *vp39* might delay the viral replication cycle by not entering the late phase.

This hypothesis is supported by the observation that four *lefs* (*p47*, *lef-4*, *lef-11* and *dnapol*) are significantly up regulated (Fig 5.7A, H, K, N). Viral *lefs* are considered as early genes and their gene products are essential for viral late gene expression (Passarelli and Miller, 1993b; Todd *et al.*, 1995). Briefly, (as LEFs are described elsewhere in more detail: Introduction 1.5.5; Chapter 4), DNAPol is essential for viral replication, whereas both *p47* and LEF-4 are parts of the viral RNA polymerase (Tomalski *et al.*, 1988; Carstens *et al.*, 1993; Todd *et al.*, 1995; Jin *et al.*, 1998). The precise role for LEF-11 is still unclear but it is potentially involved in viral replication (Lin and Blissard, 2002; Dong *et al.*, 2017).

Interestingly, expression of *p15* is also enhanced (Fig. 5.7F). Its function has been only characterised in BmNPV, in which it encodes for a nonessential capsid protein (Lu and Iatrou, 1997; Ono *et al.*, 2012). Therefore, it could be speculated that *p15* gets overexpressed to partially compensate for the loss of the major capsid protein.

Moreover, transcription of the two envelope glycoproteins *gp64* and *gp41* were increased after *vp39* RNAi-treatment (Fig. 5.7S, V). The envelope of BVs contains the viral glycoprotein GP64 that mediates pH-dependent membrane fusion during virus entry. Additionally, it is a limiting factor for BV production and required for infectivity of BV, which spreads infection from cell to cell and mediates the systemic infection of host cells (Blissard and Wenz, 1992; Monsma *et al.*, 1996; Hefferon *et al.*, 1999; Oomens and Blissard, 1999). On the other side, GP41 was found in ODVs but not BVs and is a

structural glycoprotein that contains a putative O-linked GlcNAc glycosylation site (Whitford and Faulkner, 1992). However, cells infected with a virus containing a ts mutant of *gp41* were not able to produce BVs at the non-permissive temperature. Nucleocapsids were produced but retained in the nucleus suggesting a defect in their egress (Olszewski and Miller, 1997). One could speculate that the increased production of the glycoproteins and the simultaneous reduction of the major capsid protein may increase the fitness of the remaining virions by the enhanced incorporation of the envelope proteins.

The speculation that the virus may try to increase its fitness after the loss of VP39 might be further supported by the observation that expression of two viral PIFs (*orf83* and *orf115*) were up regulated, which are essential for oral infectivity *in vivo*. The *orf83* encodes for VP91 and *orf115* for PIF-3 (Fig. 5.7L, T; Ohkawa *et al.*, 2005; Zhu *et al.*, 2013; Javed *et al.*, 2016).

Another viral gene that was up regulated after *vp39* RNAi was *orf11* (Fig. 5.7E). Knock-out of the early viral gene *orf11* resulted in the loss of BV production in *Sf9* cells but had no effect on viral DNA replication as determined by qPCR. Additionally, electron microscopy studies demonstrated that the same mutant had a defect in the egress of the nucleocapsids from the nucleus to the cytoplasm and did not envelope the capsids in the nucleus to form ODVs. These effects were rescued by a repair virus (Tao *et al.*, 2015). This may further corroborate the hypothesis that *vp39* expression is regulated by feedback loops.

In addition, expression of a number of viral genes (*orf4*, *orf20*, *orf30*, *orf34*, *orf58*, *orf79*, *orf102*, *arif-1*, *da41* and *egt*) were up regulated after transfection of *vp39* dsRNAs (Fig. 5.4B, 5.7), which have not been associated with the major capsid protein or nucleocapsid assembly yet.

The viral ORF34 protein is localised in both the cytoplasm and the nucleus of AcMNPV infected cells. It does not contain a nuclear localisation signal (NLS) but a potential C3H zinc finger motif that might be required for its transport into the nuclear. Viruses with mutations in this motif localised only in the cytoplasm of virus-infected cells. Moreover, several mutations in *orf34* decreased BV production (Qiu *et al.*, 2017).

The early viral gene *arif-1* encodes together with *orf20* for a 45 kDa protein, which was able to induce actin rearrangements at 3 to 7 h p.i when its promoter was activated by IE-1 (Roncarati and Knebel-Mörsdorf, 1997; Harrison and Bonning, 2003).

The highly conserved viral gene *da41* did not seem to be required for either BV or ODV production nor for the formation of the viral nucleocapsid when *Sf9* cells were transfected

with a *da41* knock-out virus and compared with AcMNPV-transfected cells. However, bioassays in *T. ni* larvae at 24 h showed higher LD₅₀ values when infected with the *da41* knock-out virus, as compared with AcMNPV indicating *da41* may be required for efficient infection *in vivo* (Wang *et al.*, 2007).

The viral *egt* gene encodes an ecdysteroid UDP-glucosyl transferase that catalyses the transfer of a uridine 5'-diphosphate (UDP)-glucose to the insect moulting hormone ecdysteroid. The virus interfered with *S. frugiperda* larval development by inhibiting moulting. Larvae infected with a virus in which a *lacZ* gene replaced the viral *egt* gene moulted from fourth to fifth instar larvae, whereas only 1 of 16 wt-infected larvae moulted in the same experiments (O'Reilly and Miller, 1989).

The gene products of the viral *orf30*, *orf58*, *orf79* and *orf102* were identified as proteins potentially associated with ODVs (Braunagel *et al.*, 2003).

The precise role for *orf4* is still unclear but it does not seem to be essential for the viral replication cycle (Ono *et al.*, 2012).

It would be of further interest if the *vp39* knock-down or in general, how the transfection of a non-specific dsRNA might affect host gene expression.

Interestingly, the transfection of a control dsRNA (*lacZ*) had only minor effects on viral gene expression (Fig. 5.8). The most dramatic effect on viral gene expression was seen for *orf107* with a FC_{log2} of -2 down regulation of gene expression (Fig. 5.8). The ORF107 consists of a viral protein with an unknown function. It is likely that *orf107* together with *orf106* encodes for a single viral gene (Harrison and Bonning, 2003). However, the RPKM value of *orf107* was only 819 (Table 5.3) in virus-infected cell not treated with RNA at 24 h p.i., whereas the average RPKM value of viral genes was 8,272 suggesting no big impact of *orf107* on viral infection. Moreover, *dbp* and *egt* expression was only slightly down regulated (FC_{log2} ~ -0.5). This suggested that transfection of a dsRNA had only minor effects on viral gene expression.

Overall, the effect of a knock-down on the viral transcriptome must be seen in the context of a temporally regulated transcriptional network, in which affected genes in turn regulate expression of even more genes in transcriptional cascades. Also, it should be kept in mind that RNA-seq analysis only provides a snapshot of the gene expression network and does not analyse if the transcripts are translated or how posttranslational modifications may affect protein function.

5.7 Conclusion

This chapter aimed to examine the effect of a *vp39* knock-down on global viral transcription. Overall, a modest effect on viral gene expression was observed after *vp39* RNAi and 29 genes were considered as differentially expressed, whereby the down regulation of the very late genes *p10* and *polh* was particularly interesting. This may suggest an additional regulatory role for the major structural nucleocapsid protein. However, whether the effect seen was solely caused by *vp39* or was caused by a combination with *cg30* still needs to be determined.

Chapter Six:

Virus transcriptome analysis of an insect cell line harbouring a persistent baculovirus

Infection studies were performed by R. Arinto Garcia and R. D. Possee (Oxford Brookes University). I generated RNA-seq libraries in Garry W. Blissard's lab at BTI in the USA under a visiting scholarship program for 4.5 month in summer 2017; funded in part by Santander Scholarship. Bioinformatics analyses (data cleaning and trimming, alignment to reference genome, calculation of RPKM values) were carried out by K. Bao (BTI). I performed all downstream analyses at Oxford Brookes University.

6.1 Introduction

Baculovirus infections are highly regulated by the temporal expression of their genes (Fig. 6.1). Although the predicted gene expression model probably grossly oversimplifies the transitions between the phases of gene expression, it serves to illustrate how mRNA levels increase throughout the infection. Usually, virus infection causes the death of the insect host and the release of polyhedra upon cell lysis and disintegration of the larvae. However, the host can survive the initial infection resulting in a sublethal infection (reviewed by Bonsall *et al.*, 2005). In contrast to latent infections (e.g. Herpes Simplex Virus- HSV), virus genes are expressed and progeny virus is produced in a persistent baculovirus infection albeit at much reduced levels (Hughes *et al.*, 1997). It is thought that persistency or covert, symptomless infections in insects may play an important strategy for viruses to survive high population fluctuations over time (Burden *et al.*, 2003). However, the mechanism by which a persistent baculovirus infection is established and how the molecular mechanism of the balance between host and virus gene expression is maintained without causing the death of the insect is still unclear.

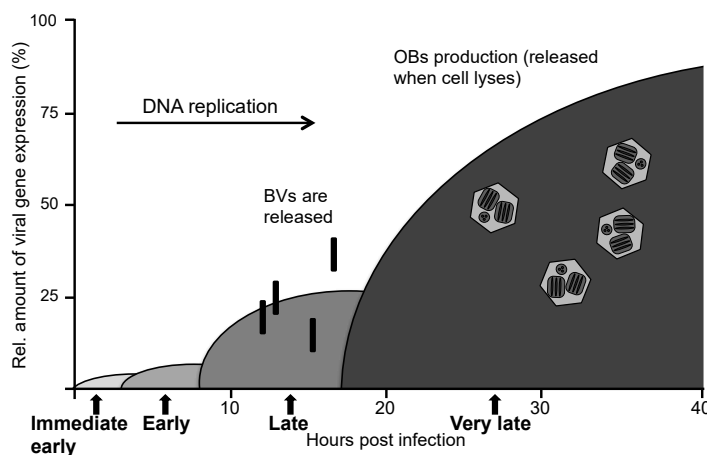


Figure 6.1 Theoretical model of temporally regulated viral gene expression in baculovirus infected cells

AcMNPV gene expression is highly regulated and genes are expressed in early, late and very late phases. During the early events, the viral infection is established and viral genes are transcribed for replication. In the late phase of infection budded viruses (BV) are produced, whereas polyhedra (OB: occlusion body) are assembled and released in the very late phase of infection after cell lysis.

Our laboratory was able to establish a persistently infected insect cell line, which was called C20. This cell line is a clonal derivative of *T. ni* Hi5 cells that became persistently infected with AcUW1.*lacZ* and has been maintained for several years. In the genome of the C20 parental virus (AcUW1.*lacZ*), the coding sequence of *p10* was replaced with *lacZ* (Weyer *et al.*, 1990). Unique for C20 cells is their unusually prolonged viability and their resilience. They can survive up to 40 days before they need to be subcultured. Moreover, they continuously produce low levels of BVs, which are infectious in *Sf* and *T. ni* Hi5 cells.

However, both cell viability and levels of BV varied between different cultures and passages. This indicates a dynamic relation between the virus and the host (R. A. Garcia, R. D. Possee, personal communication).

Genome sequencing of C20-derived virus genomes revealed minor gene deletions had occurred at some point in its evolution. The deletions included the 5' end of *egt* (encoding for an insect virus moulting protein) encompassing the promoter region of *da26* (*orf16*). The precise role of Da26 is still unclear but it has been suggested that it directly interacts with IE-0 and IE-1 (Kang *et al.*, 2005; Nie *et al.*, 2009). Another deletion was found in the *p95* (*orf83*) gene that encodes for a structural protein. In addition, *p95* was found to harbour an insertion, which resulted in the addition of a premature stop codon. The *lacZ* gene was also lost in the persistent virus. Moreover, a frameshift mutation in the *fp25k* gene was detected resulting in the introduction of a stop codon and a truncation of the protein (D. Leite, R. A. Garcia, personal communication).

One phenotype that was observed in the context of the C20 cell line was that of virus interference of superinfection. When C20 cells were challenged with AcMNPV only a minor subpopulation of C20 cells were infected with the majority of cells resistant to infection (R. Arinto Garcia, R. D. Possee, personal communication).

Interestingly, the baculovirus is maintained as a persistent infection in C20 cells. The cells do not lyse as a result of virus infection and the virus may not enter the very late phase of infection. Therefore, the C20 cells provide a unique model to determine which factors are required for the transition between late and very late phase of baculovirus infection. To gain a detailed and comprehensive picture of viral and host gene expression in C20 cells, strand-specific RNA-sequencing was performed on C20 cells and C20 cells challenged with AcMNPV. Results were compared to AcMNPV-infected *T. ni* Hi5 cells.

6.2 Strand-specific library generation for RNA-sequencing and Data analysis

Unchallenged or AcMNPV-infected (MOI 5) infected C20 (10^6 cells) or *T. ni* Hi5 cells (0.8×10^6 cells) that were seeded the evening before infection in 35 mm dishes were harvested at the indicated time points (Fig. 6.2A; R. Arinto Garcia, R. D. Possee). Total RNA was extracted using TRIzol® (Invitrogen™) and RNA degradation and contamination was examined using 1% (w/v) agarose gels stained with ethidium bromide (data not shown). Strand-specific RNA-seq libraries using 4 µg of total RNA were generated as described before and multiplexed as indicated in Table 6.1 (Fig. 6.2B; Chapter 5.1; Zhong *et al.*, 2011; Chen *et al.*, 2013). In total, 19 libraries were pooled per Illumina® RNA-seq lane (Table 6.1).

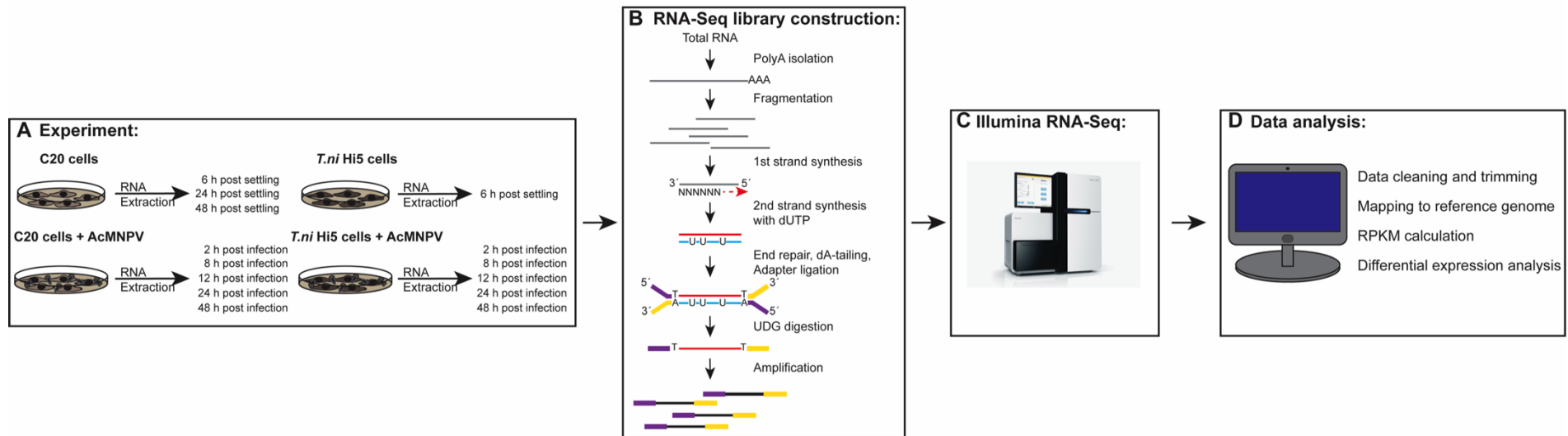


Figure 6.2 C20 RNA-seq experimental design

A) Total RNA of C20 cells and *T. ni* Hi5 cells or AcMNPV challenged C20 or *T. ni* Hi5 cells was extracted at the indicated time points. B) Strand-specific libraries for RNA-seq were generated as previously described (Zhong *et al.*, 2011). C) Sequencing was outsourced (Illumina®, Weill Cornell Medical School). D) Raw reads were cleaned and trimmed and mapped to the AcMNPV genome and the reads per kilo base per million mapped reads (RPKM) values were calculated (K. Bao, BTI).

Table 6.1 C20 samples for Illumina® RNA-seq: Pooled libraries with index sequences

Pooled libraries no 1, final concentration 10.2 ng/μl in 21 μl TE buffer		
Sample	Illumina® index	Index sequence
<i>T. ni</i> Hi5 mock 1	1	CGTGAT
<i>T. ni</i> Hi5 mock 2	2	ACATCG
<i>T. ni</i> Hi5 mock 3	3	GCCTAA
<i>T. ni</i> Hi5 2 h p.i. 1	4	TGGTCA
<i>T. ni</i> Hi5 2 h p.i. 2	5	CACTGT
<i>T. ni</i> Hi5 2 h p.i. 3	6	ATTGGC
<i>T. ni</i> Hi5 8 h p.i. 1	7	GATCTG
<i>T. ni</i> Hi5 8 h p.i. 2	8	TCAAGT
<i>T. ni</i> Hi5 8 h p.i. 3	9	CTGATC
<i>T. ni</i> Hi5 12 h p.i. 1	10	AAGCTA
<i>T. ni</i> Hi5 12 h p.i. 2	11	GTAGCC
C20 18 h p.i. 2	44	ATTATA
C20 24 h p.i. 1	46	TCGGGA
<i>T. ni</i> Hi5 18 h p.i. 2	14	GGAACT
<i>T. ni</i> Hi5 18 h p.i. 3	15	TGACAT
<i>T. ni</i> Hi5 24 h p.i. 1	41	GTCGTC
<i>T. ni</i> Hi5 24 h p.i. 2	17	CTCTAC
C20 24 h p.i. 2	20	GGCCAC
<i>T. ni</i> Hi5 48 h p.i. 1	43	GCTGTA
Pooled libraries no 2, final concentration 10.1 ng/μl in 21 μl TE buffer		
Sample	Illumina® index	Index sequence
<i>T. ni</i> Hi5 48 h p.i. 2	20	GGCCAC
<i>T. ni</i> Hi5 48 h p.i. 3	21	CGAAAC
C20 mock 6 h 1	22	CGTACG
C20 mock 6 h 2	44	ATTATA
C20 mock 6 h 3	24	GCTACC
C20 mock 24 h 1	1	CGTGAT
C20 mock 24 h 2	2	ACATCG
C20 mock 24 h 3	27	AGGAAT
C20 mock 48 h 1	4	TGGTCA
C20 mock 48 h 2	5	CACTGT
C20 mock 48 h 3	6	ATTGGC
C20 2 h p.i. 1	7	GATCTG
C20 2 h p.i. 2	8	TCAAGT
C20 2 h p.i. 3	9	CTGATC
C20 8 h p.i. 1	10	AAGCTA
C20 8 h p.i. 2	11	GTAGCC
C20 8 h p.i. 3	12	TACAAG
C20 12 h p.i. 1	13	TTGACT
C20 12 h p.i. 2	14	GGAACT
Pooled libraries no 3*, final concentration 7.15 ng/μl in 21 μl TE buffer		
Sample	Illumina® index	Index sequence
C20 12 h p.i. 3	15	TGACAT
C20 18 h p.i. 1	16	GGACGG
<i>T. ni</i> Hi5 12 h p.i. 3	12	TACAAG
C20 18 h p.i. 3	45	GAATGA
<i>T. ni</i> Hi5 18 h p.i. 1	13	TTGACT
<i>T. ni</i> Hi5 24 h p.i. 3	18	GCGGAC
C20 24 h p.i. 3	21	CGAAAC
C20 48 h p.i. 1	22	CGTACG
C20 48 h p.i. 2	23	CCACTC
C20 48 h p.i. 3	24	GCTACC

*Samples (total Σ 19) were pooled with different project (A. Shrestha, BTI)

The quality of purified and pooled libraries was assessed by agarose gel electrophoresis (Fig. 6.3). The mean size of DNA fragments in each of the pooled libraries was between 200-300 bp. The optimal size of DNA fragments was determined by the limitations of the NGS platform, which for Illumina® instruments is in general <500 bp with a peak of ~250-300 bp (Illumina Sequencing Manual). Illumina® RNA-seq was outsourced to Epigenomic

core using the Illumina® HiSeq4000 platform (Fig. 6.2C; Weill Cornell Medical School, NYC, US) generating at least 15 million reads on average per library (Chapter 5.1).

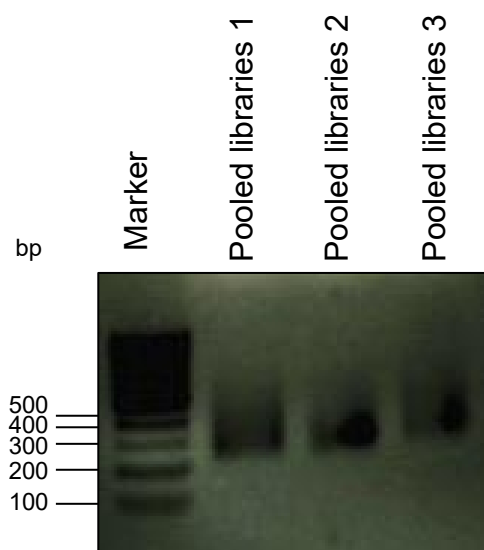


Figure 6.3 Agarose gel of pooled RNA-seq libraries

To analyse the length distribution of the DNA fragments from the pooled libraries, 2 µl of purified pooled libraries were run on a 1% (w/v) agarose gel stained with ethidium bromide.

Illumina® raw reads were de-multiplexed then adapters, poly A/T and rRNA sequences were removed (Fig. 6.2D; Table 6.2; K. Bao). The library “C20 18 h p.i. replicate no 2” received only ~1,000 raw reads instead of the expected ~15 millions for unknown reasons. Hence, this sample was subsequently removed from any downstream analysis. Cleaned reads were mapped against the tetra- and alphanodavirus genome as a quality control and to remove those sequences (Table 6.2; Chapter 5.1; K. Bao). As expected, no reads could be mapped against the tetravirus genome (Table 6.2) but several hundreds to thousands of reads per library were mapped to the alphanodavirus genome for both cell lines *T. ni* Hi5 and its clonal derivative C20 either unchallenged or infected with AcMNPV (Table 6.2; K. Bao). Quality control after the final cleaning of the raw reads and the removal of alphanodavirus sequences revealed that each library received about 21.9 million reads on average, whereby 13 million reads was the lowest number of filtered reads obtained (Table 6.2).

Filtered reads were mapped to the AcMNPV genome (Fig. 6.4A; supplementary CD Table S 6.1; Accession no. NC_001623; K. Bao; Ayres *et al.*, 1994; Chen *et al.*, 2013). Only 0.3% of the cleaned reads could be mapped to the virus genome in AcMNPV-infected *T. ni* Hi5 at 2 h p.i., which just slightly increased during the first 12 h p.i. However, this value dramatically increased to 95% at 48 h p.i. On the contrary, only ~2% of the filtered reads could be mapped to the AcMNPV genome in unchallenged C20 cells at either time point post settling (p. s.; Fig. 6.4B) indicating that virus transcription occurred at low levels.

However, when C20 cells were challenged with AcMNPV, the number of mapped reads to the virus genome increased after a lag phase of about 12 h up to 4.2% at 48 h p.i. suggesting that a subpopulation of cells could be superinfected (Fig. 6.3C').

After filtered reads were mapped to the virus, RPKM values were calculated (K. Bao; Supplementary CD Table S 6.2). Viral RPKM values were adjusted as described previously (Chapter 5.3; Supplementary CD Table S 6.1; Appendix Fig. S 6.1-6.15; Chen *et al.*, 2013). Any negative gene expression values resulting from RPKM adjustments were considered as 0 (Chapter 5.3).

Unfortunately, expression of the following transcripts could not be determined accurately: *orf44*, *orf45*, *etm*, *orf56*, *lef-10*, *orf78*, *orf81*, *orf113* and *alk-exo*. Their transcription levels could not be resolved separately as they share 3'transcriptional co-termination sites with adjacent genes and/or their mRNAs overlap with adjacent genes to a large extent (supplementary CD Table S 6.2; Chen *et al.*, 2013). Therefore, those genes were removed from any further downstream analysis.

6.3 Virus gene transcription in C20 cells

A characterisation of virus persistence that is distinct from latency is that virus genes are expressed at low levels and there is some production of BVs. Illumina® RNA-seq analysis confirmed that all virus genes were expressed in unchallenged C20 cells (supplementary CD Table S 6.1; S 6.2). Although gene expression of *HisP* in C20 cells could not be detected at any time p.s., resequencing of the AcMNPV genome suggested that *HisP* together with *v-fgf* encodes for a single gene (Harrison and Bonning, 2003). Transcription of *v-fgf* could be observed for C20 cells at 6 h p.s. (supplementary CD Table S 6.2).

6.4 Virus gene transcription in superinfected C20 cells

To confirm if C20 cells were superinfected with AcMNPV (Fig. 6.4C'), *p10* expression was used as a marker to follow infection. The virus utilised for the establishment of the C20 cell line was AcUW1.*lacZ*, in which *lacZ* replaced parts of the *p10* coding region. The *lacZ* coding sequence was inserted into the *Bgl*II site of *p10*, while the first 152 nt of *p10* were deleted. Thus, AcUW1.*lacZ* still contained the *p10* 5'leader sequence, 132 remaining nt at the 3'end and the 3'UTR (Weyer *et al.*, 1990). Therefore, in unchallenged C20 samples, a few reads could be mapped to *p10* (supplementary CD Table S 6.1, S 6.2). However, when C20 cells were superinfected with AcMNPV, *p10* expression increased ~160-fold at 48 h p.i. compared to unchallenged C20. Moreover, the *p10* expression pattern in C20 cells resembled that of AcMNPV-infected *T. ni* Hi5 cells with a lag phase of about 18 h, while highest expression levels occurred very late during virus infection at 48 h p.i. indicating that some C20 cells could be infected (Fig. 6.4C', 6.5).

Table 6.2 Illumina® RNA-seq C20 data cleaning summary

sample	hour	total raw reads	remove adapter, low quality		remove polyA/T		remove rRNA		clean	remove Tetravirus		remove Alphanodovirus		final clean
			clean	%of raw	No. polyA/T	%polyA/T	No. reads	%rRNA		No. Tet	%Tet	No. Alph	%Alph	
C20	mock 6 h	21,025,987	20,574,593	97.85	156,776	0.76	36,506	0.18	20,381,311	0.00	0.00	1,214	0.01	20,380,097
		23,893,804	23,541,995	98.53	77,504	0.33	185,289	0.79	23,279,202	0.00	0.00	1,330	0.01	23,277,872
		22,829,450	22,510,334	98.60	20,957	0.09	103,525	0.46	22,385,852	0.00	0.00	860	0.00	22,384,992
	mock 24 h	20,699,936	20,356,495	98.34	26,887	0.13	129,143	0.64	20,200,465	0.00	0.00	643	0.00	20,199,822
		22,731,908	22,266,836	97.95	46,228	0.21	131,800	0.59	22,088,808	0.00	0.00	741	0.00	22,088,067
		18,335,927	16,470,692	89.83	290,363	1.76	52,780	0.33	16,127,549	0.00	0.00	823	0.01	16,126,726
	mock 48 h	18,128,498	17,870,101	98.57	10,627	0.06	82,491	0.46	17,776,983	0.00	0.00	852	0.00	17,776,131
		19,761,127	19,389,951	98.12	39,459	0.20	63,575	0.33	19,286,917	0.00	0.00	876	0.00	19,286,041
		20,227,857	19,913,030	98.44	21,878	0.11	80,692	0.41	19,810,460	0.00	0.00	1,128	0.01	19,809,332
	2 h p.i.	23,895,233	23,057,849	96.50	182,063	0.79	54,411	0.24	22,821,375	0.00	0.00	822	0.00	22,820,553
		17,351,447	17,092,440	98.51	18,604	0.11	130,073	0.76	16,943,763	0.00	0.00	403	0.00	16,943,360
		17,889,614	17,547,213	98.09	6,430	0.04	193,304	1.10	17,347,479	0.00	0.00	442	0.00	17,347,037
	8 h p.i.	20,356,006	18,113,745	88.98	36,656	0.20	82,400	0.46	17,994,689	0.00	0.00	706	0.00	17,993,983
		17,766,534	17,402,267	97.95	29,108	0.17	135,701	0.78	17,237,458	0.00	0.00	613	0.00	17,236,845
		19,662,096	19,391,690	98.62	12,986	0.07	138,323	0.71	19,240,381	0.00	0.00	697	0.00	19,239,684
	12 h p.i.	19,198,822	18,899,894	98.44	18,768	0.10	121,479	0.64	18,759,647	0.00	0.00	673	0.00	18,758,974
		17,199,717	16,979,885	98.72	4,592	0.03	143,169	0.84	16,832,124	0.00	0.00	604	0.00	16,831,520
		22,619,951	22,255,761	98.39	10,236	0.05	168,425	0.76	22,077,100	0.00	0.00	970	0.00	22,076,130
	18 h p.i.	20,812,817	20,535,135	98.67	10,449	0.05	171,595	0.84	20,353,091	0.00	0.00	724	0.00	20,352,367
		1,322	1,238	93.65	31	2.50	8	0.66	1,199	0.00	0.00	0	0.00	1,199
		27,167,241	26,682,579	98.22	41,508	0.16	257,089	0.97	26,383,982	0.00	0.00	781	0.00	26,383,201
	24 h p.i.	14,949,212	14,289,818	95.59	352,090	2.46	91,145	0.65	13,846,583	0.00	0.00	365	0.00	13,846,218
		20,496,231	20,210,514	98.61	12,706	0.06	176,699	0.87	20,021,109	0.00	0.00	659	0.00	20,020,450
		29,133,662	28,710,303	98.55	18,875	0.07	252,177	0.88	28,439,251	0.00	0.00	1,158	0.00	28,438,093
	48 h p.i.	25,498,823	25,130,790	98.56	11,242	0.04	205,494	0.82	24,914,054	0.00	0.00	1,235	0.00	24,912,819
		35,027,399	34,550,471	98.64	14,237	0.04	285,974	0.83	34,250,260	0.00	0.00	1,909	0.01	34,248,351
		27,966,881	27,561,122	98.55	29,797	0.11	148,327	0.54	27,382,998	0.00	0.00	1,789	0.01	27,381,209

Table 6.2 Continued: Illumina® RNA-seq C20 cleaning summary

sample	hour	total raw reads	remove adapter, low quality		remove polyA/T		remove rRNA		clean	remove Tetravirus		remove Alphanodavirus		final clean
			clean	%of raw	No. polyA/T	%polyA/T	No. reads	%rRNA		No. Tet	%Tet	No. Alph	%Alph	
<i>T. ni</i> Hi5	mock 6 h	17,320,371	17,051,167	98.45	26,953	0.16	164,797	0.97	16,859,417	0.00	0.00	210	0.00	16,859,207
		21,824,904	21,485,415	98.44	24,734	0.12	233,469	1.09	21,227,212	0.00	0.00	235	0.00	21,226,977
		16,662,840	16,412,805	98.50	14,333	0.09	181,985	1.11	16,216,487	0.00	0.00	228	0.00	16,216,259
	2 h p.i.	22,779,000	22,415,665	98.40	54,453	0.24	132,596	0.59	22,228,616	0.00	0.00	272	0.00	22,228,344
		15,594,391	14,472,558	92.81	549,745	3.80	8,173	0.06	13,914,640	0.00	0.00	128	0.00	13,914,512
		22,217,241	21,875,455	98.46	19,382	0.09	270,146	1.24	21,585,927	0.00	0.00	190	0.00	21,585,737
	8 h p.i.	26,525,297	26,077,964	98.31	43,320	0.17	152,235	0.58	25,882,409	0.00	0.00	338	0.00	25,882,071
		20,136,274	19,821,492	98.44	34,757	0.18	201,534	1.02	19,585,201	0.00	0.00	330	0.00	19,584,871
		20,578,250	20,232,862	98.32	26,878	0.13	135,325	0.67	20,070,659	0.00	0.00	320	0.00	20,070,339
	12 h p.i.	32,077,478	31,303,384	97.59	67,703	0.22	242,725	0.78	30,992,956	0.00	0.00	602	0.00	30,992,354
		17,304,983	16,409,582	94.83	562,362	3.43	8,594	0.05	15,838,626	0.00	0.00	120	0.00	15,838,506
		31,612,597	31,083,063	98.32	55,638	0.18	175,089	0.56	30,852,336	0.00	0.00	580	0.00	30,851,756
	18 h p.i.	34,435,997	33,903,968	98.46	42,512	0.13	136,747	0.40	33,724,709	0.00	0.00	1,062	0.00	33,723,647
		19,522,001	19,238,556	98.55	15,586	0.08	115,498	0.60	19,107,472	0.00	0.00	622	0.00	19,106,850
		17,398,266	16,498,867	94.83	138,429	0.84	9,877	0.06	16,350,561	0.00	0.00	233	0.00	16,350,328
	24 h p.i.	17,016,208	16,678,920	98.02	148,593	0.89	46,649	0.28	16,483,678	0.00	0.00	1,132	0.01	16,482,546
		32,351,955	31,891,663	98.58	20,803	0.07	204,533	0.64	31,666,327	0.00	0.00	1,483	0.00	31,664,844
		24,587,121	24,245,481	98.61	9,464	0.04	95,765	0.40	24,140,252	0.00	0.00	1,915	0.01	24,138,337
	48 h p.i.	18,990,370	18,639,192	98.15	114,177	0.61	15,246	0.08	18,509,769	0.00	0.00	64,834	0.35	18,444,935
		14,997,931	13,868,913	92.47	700,311	5.05	899	0.01	13,167,703	0.00	0.00	3,365	0.03	13,164,338
		15,820,309	15,463,024	97.74	124,491	0.81	6,337	0.04	15,332,196	0.00	0.00	21,078	0.14	15,311,118

Abbreviations: Tet: tetravirus, Alph: alphanodavirus, polyA/T: polyadenylated, adenine/thymine

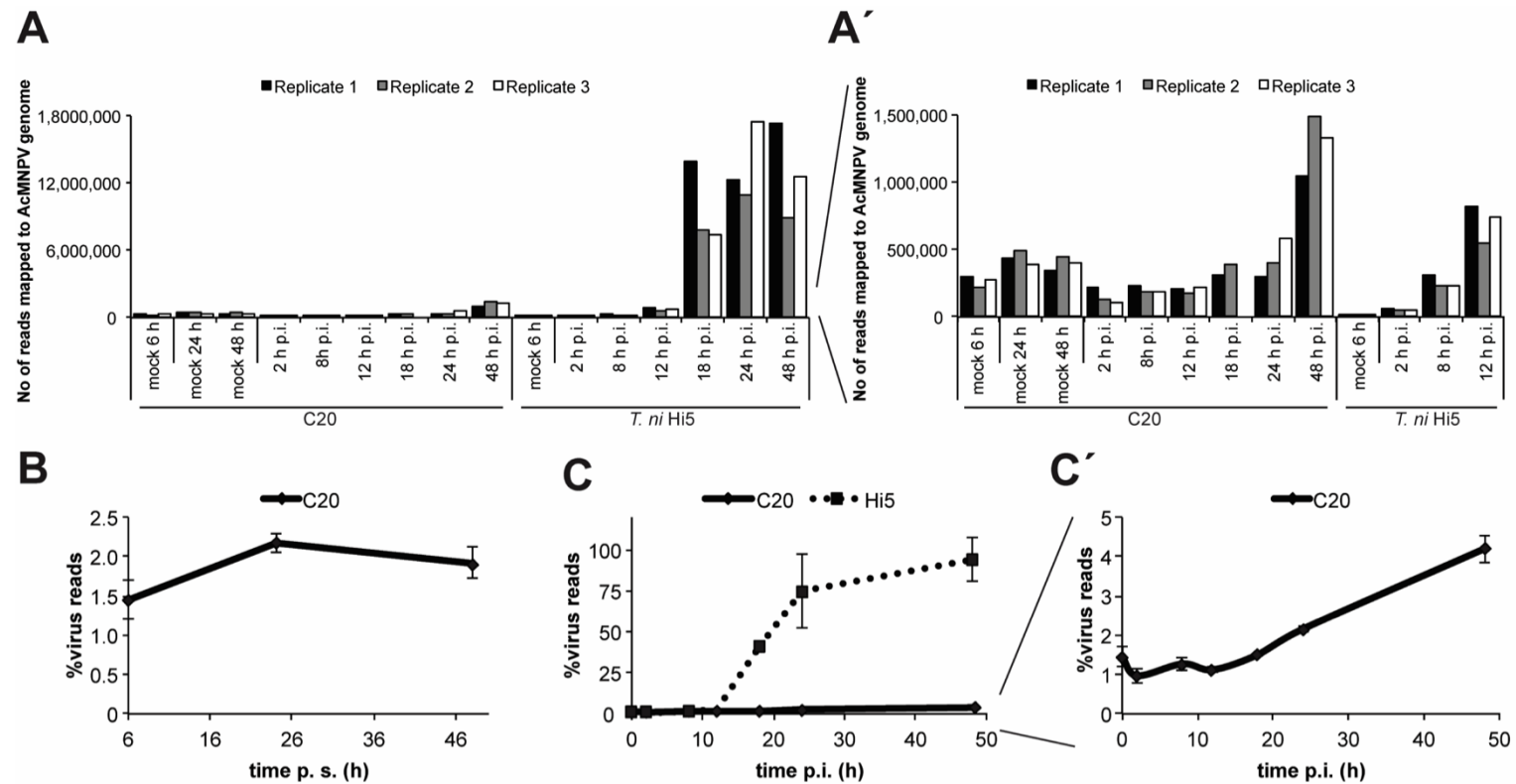


Figure 6.4 Sequencing statistics

Total number of Illumina® sequencing reads for each replicate that was mapped to the A) AcMNPV genome. A') represents an enlargement of A. Average percentage of reads (as % of cleaned reads) in B) unchallenged C20 or C) AcMNPV-infected C20 and *T. ni* Hi5 cells that were mapped to the viral genome. C') represents an enlargement of C.

However, caution should be taken by directly comparing RPKM values between AcMNPV-infected *T. ni* Hi5 or C20 cells. The reason for this is that gene expression was normalised as RPKM values, whereby the sequencing depth (read number) contained both virus and host read numbers. While overall less virus reads were observed for all C20 samples, virus reads for infected *T. ni* Hi5 cells increased dramatically until 48 h p.i. (Fig. 6.4). This could have been overcome by normalising virus gene expression with virus reads only.

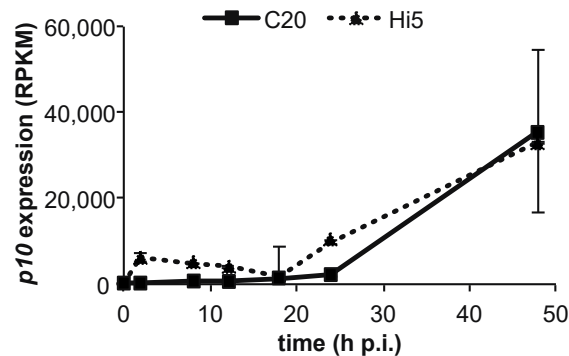


Figure 6.5 *p10* expression profile in AcMNPV-infected cells C20 and *T. ni* Hi5 cells

Normalised *p10* expression (RPKM) in AcMNPV-infected *T. ni* Hi5 (Hi5) and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.

The expression of *p10* in superinfected C20 cells was confirmed by running cell lysates on a protein gel, which was subsequently stained by Coomassie blue. While no P10 accumulation was detected for unchallenged C20 cells, superinfected C20 cells accumulated P10 although not to the extent observed in AcMNPV-infected *T. ni* Hi5 cells (R. Arinto Garcia, personal communication).

6.5 Most abundant viral transcripts

To illustrate the temporal expression of viral genes in AcMNPV-infected *T. ni* Hi5 cells, the six most abundant expressed genes throughout the time course of infection were determined (Table 6.3). At 2 h p.i., the immediate early genes *pe38* (20% of total viral mRNA), *ie-2* (14%) and *ie-1* (4%) were three of the six highest mRNAs present. The transcripts of *p6.9* (18%), *me53* (7%) and *polh* (7%) were the other three abundant mRNAs. At 8 h p.i. the most abundant transcripts were *lef-3* (11%), *pp31* (9%), *dbp* (10%), *pk-2* (5%) and *gp64* (4%). From 12 h p.i. *p6.9* was by far the highest expressed gene representing more than half of all viral transcripts by 24 h p.i. At 18 h p.i., viral transcripts from *orf76*, *orf75*, *orf58*, *orf73* and *orf74* were the most abundantly present. By 48 h p.i., the very late gene *polh* represented 40% of all viral transcripts. Although the second very late gene *p10* was one of the most abundant expressed mRNAs in virus-infected *T. ni* Hi5 cells at 48 h p.i., it surprisingly made up only 2% of the viral transcripts (Table 6.3).

In the persistently infected C20 cell line, *p6.9* transcripts were by far the most abundant (>32% of total viral transcripts; Table 6.3) followed by *orf76* (7%). The other most abundant viral transcripts (~2-3%) in C20 cells included: *Ac-bro* and *v-ubi*, *orf58* and *ctx odv-ec27* and *polh*. When C20 cells were superinfected with AcMNPV, it did not change the presence of the most highly abundant transcript at any time point p.i. (Table 6.3). The only difference that could be observed was the proportion of *p6.9* mRNAs, which was reduced from ~32% of total viral transcripts in unchallenged C20 cells to 17% at 24 h p.i. This subsequently increased again to 28% at 48 h p.i.

6.6 Global viral transcription patterns

The analysis of the most abundantly expressed viral genes in C20 cells would indicate that their expression in both unchallenged and superinfected cultures did not vary greatly over time (Table 6.3). To investigate whether or not this might be true for all viral genes, a global analysis of the viral transcriptome was performed.

At first, differential gene expression analysis (DGE) was performed in a productive infection by the comparison of the log₂ difference of normalised gene expression values of virus-infected cells at the indicated time points p.i. to AcMNPV-infected *T. ni* Hi5 cells at 2 h p.i. This revealed the landscape of viral gene expression in an overt infection over time (Fig. 6.6A). Gene expression peaked either immediate early at 2 h p.i. (e.g. *ie-1*), early between 8-12 h p.i. (e.g. *lef-3*), late between 18-24 h p.i. (e.g. *p6.9*) or very late at 48 h p.i. (e.g. *polh*).

To investigate whether viral genes were differentially expressed in unchallenged C20 cells, the log₂ of normalised gene expression values of C20 cells harvested at 24 h and 48 h were compared with the normalised gene expression values of C20 at 6 h p.s. (Fig. 6.6B). By looking at the heat map of differentially expressed genes in unchallenged C20 cells it was observed that: viral gene expression changed over time; most of viral genes were down regulated; whereas only a few viral genes were up regulated.

To determine whether or not this first impression might hold true and viral gene transcription was down regulated over the time course of unchallenged C20 cells, the proportion of up and down regulated genes with a significant change ($FC \geq 1.5$ or $FC \leq -1.5$, student's t-test, $p < 0.05$) was calculated (Table 6.4). At 24 h p.s., little difference in gene expression was detected and transcription of 93.2% of viral genes was not significantly up or down regulated. On the other hand, at 48 h p.s. 26 genes (17.7%) were significantly down regulated and 6 genes (4.1%) were up regulated indicating a dynamic in gene transcription by the persistently infected virus in C20 cells. The most dramatic effect was observed for *p74* expression ($FC = 10.2$), which encodes for a PIF (Faulkner *et al.*, 1997).

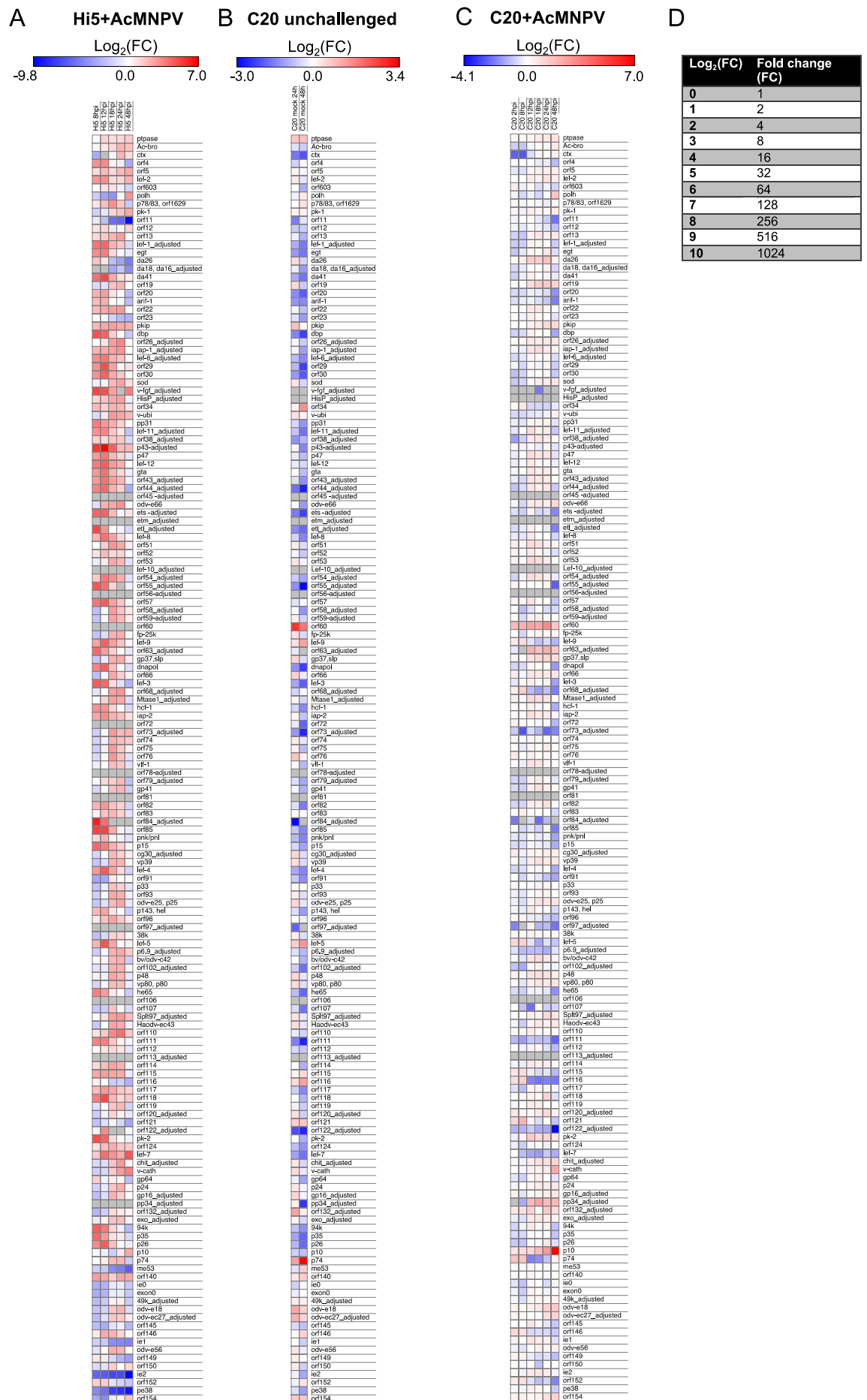
Table 6.3 Most abundant mRNAs in C20 and AcMNPV-infected C20 and *T. ni* Hi5 cells (in % of total viral mRNA)

<i>T. ni</i> Hi5 + AcMNPV											
2 h p.i.		8 h p.i.		12 h p.i.		18 h p.i.		24 h p.i.		48 h p.i.	
Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA
pe38	20	lef-3	11	p6.9	14	p6.9	44	p6.9	52	polh	40
p6.9	18	dbp	10	lef-3	8	orf76	13	orf76	6	p6.9	35
ie-2	14	pp31	9	dbp	7	orf75	3	polh	3	Ac-bro	2
me53	7	p6.9	5	orf82	5	orf58	2	odv-e25	3	p10	2
polh	7	pk-2	5	pp31	5	orf73	2	Ac-bro	2	orf74	2
ie-1	4	gp64	4	p26	4	orf74	2	ctx	2	orf73	2

unchallenged C20						C20 + AcMNPV											
6 h p.s.		24 h p.s.		48 h p.s.		2 h p.i.		8 h p.i.		12 h p.i.		18 h p.i.		24 h p.i.		48 h p.i.	
Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA	Gene	% mRNA
p6.9	38	p6.9	32	p6.9	32	p6.9	32	p6.9	28	p6.9	21	p6.9	20	p6.9	17	p6.9	28
orf76	7	orf76	12	orf76	10	orf76	11	orf76	10	orf76	8	orf76	7	orf76	8	orf76	6
Ac-bro	3	odv-ec27	3	v-ubi	4	orf58	3	odv-ec27	3	Ac-bro	3	Ac-bro	4	Ac-bro	3	polh	5
orf58	3	v-ubi	3	odv-ec27	3	orf74	3	Ac-bro	3	orf58	2	orf58	3	odv-ec27	3	Ac-bro	5
v-ubi	2	orf58	3	polh	3	Ac-bro	2	orf74	2	orf74	2	odv-e25	3	odv-e25	3	odv-ec27	4
ctx	2	Ac-bro	3	Ac-bro	3	orf75	2	v-ubi	2	odv-e25	2	ctx	3	orf82	3	v-ubi	3

Figure 6.6 Differential gene expression analysis of AcMNPV-infected *T. ni* Hi5 cells and unchallenged or superinfected C20 cells

Normalised viral gene expression (RPKM values) of A) AcMNPV-infected *T. ni* Hi5 cells at the indicated time points p.i. were compared to AcMNPV-infected *T. ni* Hi5 cells at 2 h p.i. or B) C20 at 24 or 48 h p. seeding were compared to normalised viral gene expression of C20 cells 6 h p. settling or C) C20 6 h p. settling was compared to normalised viral gene expression of superinfected C20 cells at the indicated time points and A-D) fold change (FC) of viral gene expression was presented on a log₂ scale as heat maps. (Grey indicated no detected gene expression.)



Alternatively, when the DGE as a $\log_2$ difference of superinfected C20 cells to unchallenged C20 at 6 h was calculated, the landscape of gene expression obtained contained some alterations to that observed in a productive infection (Fig. 6.6C). For example, *ie-1* expression in AcMNPV-infected C20 cells peaked later (12-18 h p.i.) than in a productive infection. The late gene *p6.9* was the most highly expressed in C20 mock cells (Table 6.3). However, *p6.9* expression decreased after superinfection (Fig. 6.6C; Table 6.3). Other genes in AcMNPV-infected C20 such as *polh* followed the same transcription pattern as in a productive infection (Fig 6.6A, C).

Table 6.4 Pairwise DGE analysis of unchallenged C20 cells*

Sample	Up regulated* (FC $\geq$ 1.5)	Down regulated* (FC $\leq$ -1.5)	Not regulated	%Up	%Down	%Not regulated
C20 unchallenged 24 h p.s.	2	8	137	1.4	5.4	93.2
C20 unchallenged 48 h p.s.	6	26	169	4.1	17.7	78.2

*DGE compared to unchallenged C20 cells at 6 h p.s. *Significant up or down regulated viral genes were determined by a Student's t-test (with $p < 0.05$). Abbreviations: DGE: differentially gene expression; FC: fold change

To gain an even broader understanding about how viral gene expression differed in both an overt and a persistent infection, either unchallenged or superinfected, cluster analysis of viral mRNA abundance over time was performed.

Initially, gene transcription was clustered in the traditional/historical way for baculovirus gene expression analysis into immediate early, early, late and very late genes, which was mainly based on promoter sequences (Fig. 6.7A). Genes containing a TAAG promoter motif were considered as late, whereas genes with a non-TAAG promoter motif were considered as early (Chen *et al.*, 2013). If both motifs were present, the time point of maximum transcription determined the nature of the gene. Very early (*pe38*, *ie-1*, *ie-0*, *ie-2*) and very late genes (*p10*, *polh*) were classified based on the literature (Guarino and Summers, 1986; Chisholm and Henner, 1988; Krappa and Knebel-Mörsdorf, 1991; Yoo and Guarino, 1994). For a temporal gene expression analysis, the normalised gene expression values from either immediate early (n=4), early (n=61), late (n=80) or very late (n=2) genes were summated at each time point p. i. to obtain transcription profiles (Fig. 6.7B; supplementary CD Table 6.3).

Viral gene expression increased over the time course of AcMNPV infection in *T. ni* Hi5 cells (Fig. 6.4A, C, 6.7B). At the same time, expression profiles of each cluster had the structure of a normal distribution peaking at different time points p.i. except for genes grouped into the very late phase. Transcription of immediate early genes reached a maximum at 2 h p.i. (Fig. 6.7B). Maximum transcription of genes clustered into the early phase was reached between 8-12 h p.i. (Fig. 6.7B). The largest group of early genes with

a determined function (31.1%) encoded for regulatory proteins (Fig. 6.7C). Late genes were expressed the highest between 18-24 h p.i. and ~25% of them encoded for structural proteins (Fig. 6.6B, C). Interestingly, a second, smaller maximum of genes expression of late genes could be observed at 2 h p.i. (Fig. 6.7B). Expression of the very late genes *p10* and *polh*, started to increase from 24 h p.i. although their transcription had not reached a maximum at 48 h p.i., the latest time point sampled (Fig. 6.7B).

When viral genes of either C20 or superinfected C20 cells were clustered as described before, transcription profiles altered dramatically from a lytic virus infection (Fig. 6.7D, E). Although gene expression of C20 cells changed over the observed time period, transcription profiles altered compared to an overt infection. Gene expression did not occur in the typical temporal regulated phases as in an overt baculovirus infection.

Moreover, neither of the expression clusters (immediate early, early, late or very late) in uninfected C20 cells reached a maximum at any time p.s. (Fig. 6.7D). No transition between the phases of gene expression could be observed and hence no transition between the late and the very late phase of infection occurred in C20 cells. Late genes were the most highly expressed, while immediate early and very late genes were the least transcribed. However, very late genes consisted only of *polh* transcription in C20 cells as the persistent virus contained a deletion inside the *p10* coding region.

However, expression profiles between unchallenged and superinfected C20 cells varied. Gene transcription of unchallenged C20 cells seemed to decrease over time (Fig. 6.7D; Table 6.4), while gene expression of AcMNPV-infected C20 cells decreased until 8 h p.i. and subsequently increased (Fig. 6.7E; C20 0 h p.i.: corresponds to C20 mock 6h). Also, in superinfected C20 cells no transition between phases of infection occurred. Additionally, as observed already in C20 cells, late genes were expressed the highest and immediate early and very late genes the least (Fig. 6.7E).

Cluster analysis of unchallenged C20 cells by the hierarchical complete linkage method identified three distinct groups of virus expression profiles: Group 1, Group 2 and Group 3 (Fig. 6.8A, B; supplementary CD Table S 6.4; Morpheus Software). Transcription of genes clustered into Group 1 decreased over time. More than half of the genes in this group (53.1%) contained an early promoter motif and moreover, 28.1% of them encoded for regulatory proteins (Fig. 6.8C, D). Expression of Group 2 genes reached a maximum between 0-24 h p.s., after which their expression decreased again. Transcription of genes clustered into Group 3 peaked between 24-48 h (Fig. 6.8A, B). The majority of genes of both Group 2 and 3 (Group 2: 62.1%, Group 3: 80%) contained a late promoter motif. The largest groups of genes in these clusters with a determined function encoded for structural proteins (Group 2: 27.5%, Group 3: 32.0%; Fig. 6.8C, D).

On the other hand, virus gene expression clustering of superinfected C20 cells by the hierarchical complete linkage method identified four distinct groups: Group A, Group B, Group C and Group D (Morpheus Software). While virus gene expression profiles of Group B-D mimicked the ones from a productive infection reaching a maximum at 8 h p.i., 12-18 h p.i. and after 48 h p.i., respectively, transcription of genes from Group A decreased over the time course of infection (Fig. 6.9A, B). Although transcription profiles from Group B-D showed similarities to an overt infection, genes that were clustered in these groups were different (Fig. 6.9C, D; supplementary CD Table S 6.5). While gene expression from Group B was highest at 2 h p.i., those genes mainly contained late promoter motifs (83.3%) and the largest group of those genes (apart from the unknown genes) encoded for structural protein (27.8%; Fig. 6.9C, D). Genes from Group C, which reached an expression maximum at 12 h p.i. encoded for regulatory proteins (40.3%; Fig. 6.9C, D). However, the most interesting group were genes clustered into Group A as their expression profiles were not present in an overt infection. Genes of this group contained a high number of early promoter motifs (70%) and encoded for proteins with yet unknown functions (63.2%; Fig. 6.9C, D).

6.7 Discussion

In this study, RNA-seq was utilised to characterise the virus transcriptome of an insect cell line harbouring a persistent baculovirus to determine the factors required for virus late and very late gene expression. Virus gene expression was compared to a productive virus infection. Moreover, the phenotype of superinfection exclusion of the persistent virus containing cell line was analysed in more detail.

To compare the data, a global transcriptome analysis of AcMNPV-infected *T. ni* Hi5 cells was performed at first. Cluster analysis of a productive virus infection in *T. ni* Hi5 cells confirmed that viral genes were temporally highly regulated during the time course of infection and moreover, that virus genes were expressed into four distinct classes: immediate early, early, late and very late (Fig. 6.6, 6.7A, B; Kelly and Lescott, 1981; Friesen and Miller, 1985). Temporal regulation of virus gene expression into phases achieves the functions required for the crucial steps during the AcMNPV replication cycle. These steps include the establishment of an infection, viral DNA synthesis, BV production, polyhedra production and release upon cell lysis (reviewed by Blissard and Rohrmann, 1990; Possee *et al.*, 2010; Rohrmann, 2013).

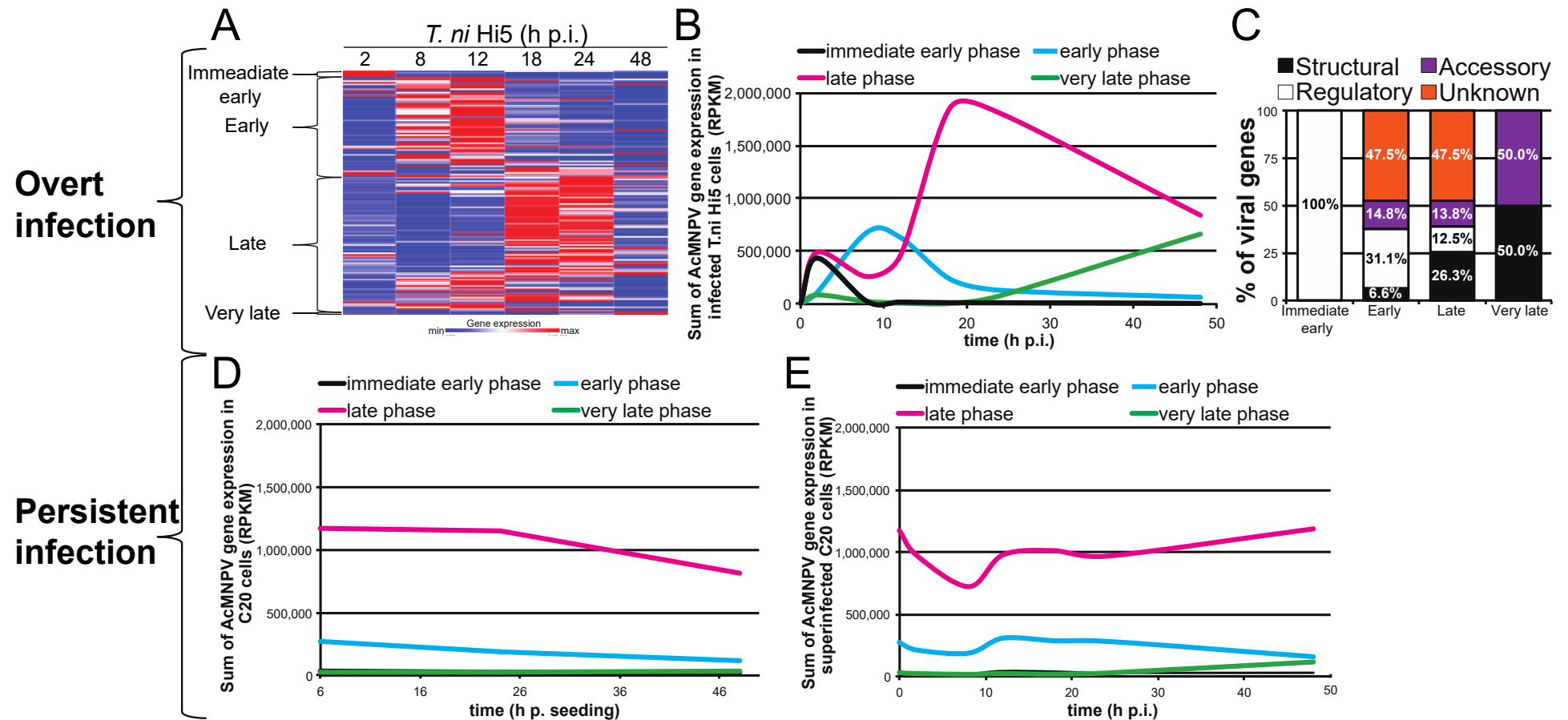


Figure 6.7 Viral gene expression in a productive infection

A) Schematics of normalised gene expression (RPKM) of AcMNPV-infected *T. ni* Hi5 cells. Normalised gene expression from all either immediate early (n=2), early (n=61), late (n=80) or very late genes (n=2) of B) AcMNPV-infected *T. ni* Hi5 cells, D) unchallenged C20 cells or E) C20 cells superinfected AcMNPV at indicated time points p.i. or p. settling was summed up to obtain viral expression profiles. C) Percentage of viral genes of AcMNPV-infected *T. ni* Hi5 cells corresponding to their function. Heat map was visualised using Morpheus Software.

Virus gene expression is usually highly temporally and/or spatially regulated. For example, HSV genes are also expressed in an immediate early, early and late phase although there is no very late phase as in baculovirus infection (Clements *et al.*, 1977). Moreover, the expression of HPV early and late genes is dependent on the differentiation of keratinocytes (reviewed by Graham, 2017).

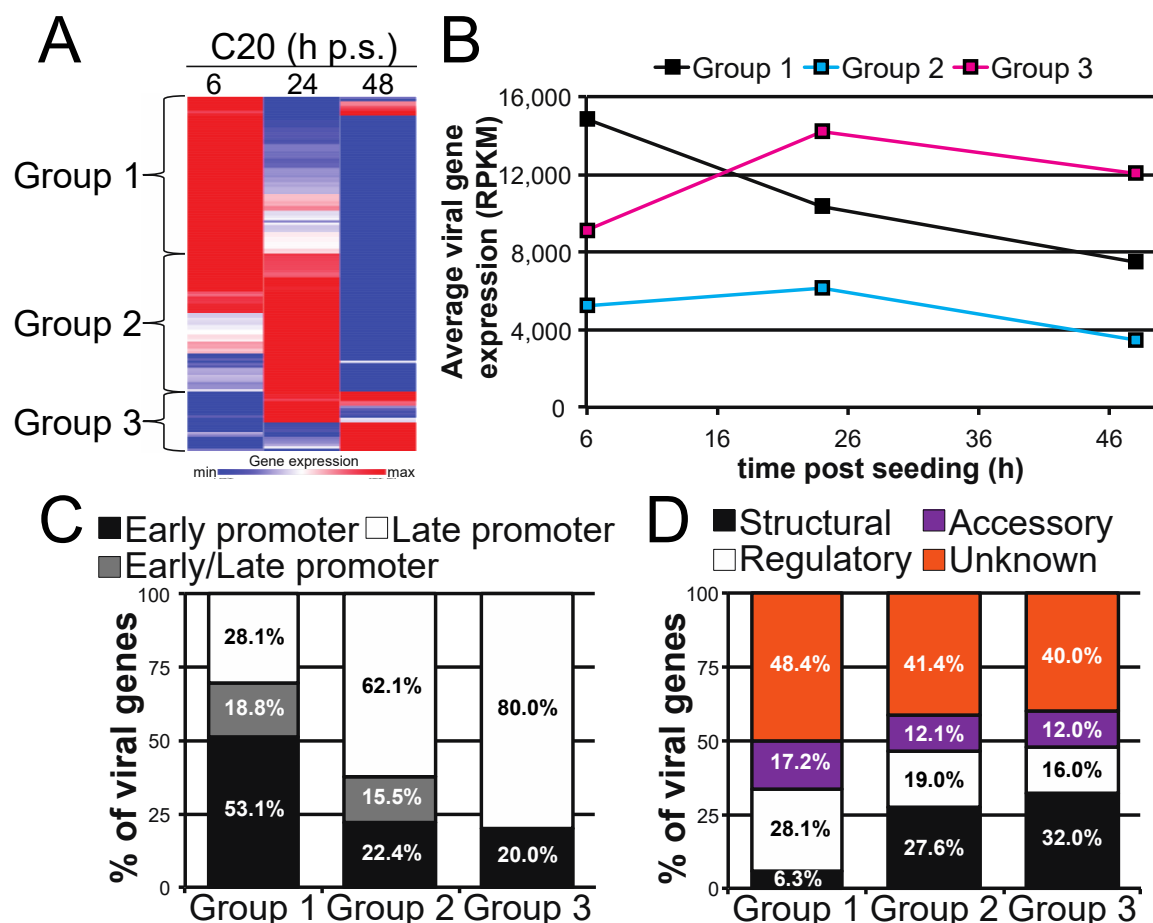


Figure 6.8 Cluster analysis of AcMNPV gene expression pattern in C20 cells

A) Schematics of normalised gene expression (RPKM) in C20 cells from 6 to 48 h p.s. Based on their gene expression pattern, viral gene expression was clustered by complete-linkage clustering into three groups: Group 1 (n=64), Group 2 (n=58) and Group 3 (n=25). B) For each group, the average gene expression pattern, as normalised gene expression levels, is shown. C-D) Percentage of viral genes corresponding to C) function and D) promoter motifs are shown. Heat map was visualised using Morpheus Software.

Expression of baculovirus immediate early genes occurred directly after virus infection with a transcription maximum reached at 2 h p.i. by exploiting the host RNA-Polymerase II (Fig. 6.6A, 6.7A, B; Table 6.3; Fuchs *et al.*, 1983). Immediate early genes (*ie-1*, *ie-0*, *ie-2* and *pe38*) encode for transcriptional regulators for early viral genes (also referred to as delayed early genes) and hence they coordinate the activation of the transcriptional cascade (Fig. 6.7C; Guarino and Summers, 1986; Krappa and Knebel-Mörsdorf, 1991; Yoo and Guarino, 1994). The major activator for viral gene expression seems to be IE-1

(Guarino and Summers, 1986), which is transcribed throughout the time course of infection (Kovacs *et al.*, 1991). Moreover, IE-1 was identified to play a role in the negative regulation of *ie-0*, *ie-2* and *pe38* transcription by interacting with an 8 bp sequence in their promoter region (Carson *et al.*, 1991b; Leisy *et al.*, 1997). Therefore, IE1 may be also involved in the shutdown of immediate early gene expression.

In addition to the immediate early genes, the transcripts of *p6.9* (18%), *me53* (7%) and *polh* (7%) were most abundantly present at 2 h p.i. (Table 6.3). This was surprising as all three of them are transcribed from late promoter motifs (Smith *et al.*, 1983c; Wilson *et al.*, 1987; Chen *et al.*, 2013) although ME53 was found as early as 6 h p.i. in virus-infected cells (de Jong *et al.*, 2009). However, they may represent transcripts that were delivered into the cells by the virus inoculum rather than being actively expressed (Chen *et al.*, 2013). As virus transcription at 2 h p.i. was very low in general (Fig. 6.4A), detection of carryover mRNAs was highly likely. Carryover transcripts may also explain the second small peak of late gene expression at 2 h p.i. (Fig. 6.7B).

Early gene expression is dependent on the activation by immediate early genes and on the host cell RNA polymerase II. The host RNA polymerase recognises specific eukaryotic promoter motifs, which are typically TAAT and CAAG motifs (Blissard and Rohrmann, 1989; Hoopes and Rohrmann, 1991; Kogan and Blissard, 1994). Transcription of early genes reached a maximum between 8-12 h p.i. (Fig. 6.7A, B). These genes mainly encoded for regulatory protein coordinating virus transcription. Among the most highly abundant genes were two *lefs* (the replicative *lef-3* and the transcriptional *lef pp31*) and *dbp* at 8 and 12 h p.i. (Fig. 6.7C; Table 6.3). The *dbp* encodes for a DNA-binding protein (Mikhailov *et al.*, 2008). In addition, these three transcripts were the most highly represented in AcMNPV-infected Tnms42 cells at 6 h p.i. (Chen *et al.*, 2013). The ssDNA-binding protein LEF-3 is part of the viral replication complex and maintained the DNA strands in a separated state during synthesis (Hang *et al.*, 1995; Taggart *et al.*, 2012). The PP31 and DBP were implicated to play a crucial role during viral DNA replication although their exact function is still unknown (Yamagishi *et al.*, 2007; Mikhailov *et al.*, 2008). Moreover, *pk-2* and *gp64* were the most abundant mRNAs at 8 h p.i. (Table 6.3). The kinase PK-2 may inhibit host stress in response to viral infection, which may be required for the establishment of a virus infection (Dever *et al.*, 1998; see Chapter 7 for details on the host transcriptome). The structural protein GP64 is incorporated into the BV and essential for virus spreading (Monsma *et al.*, 1996).

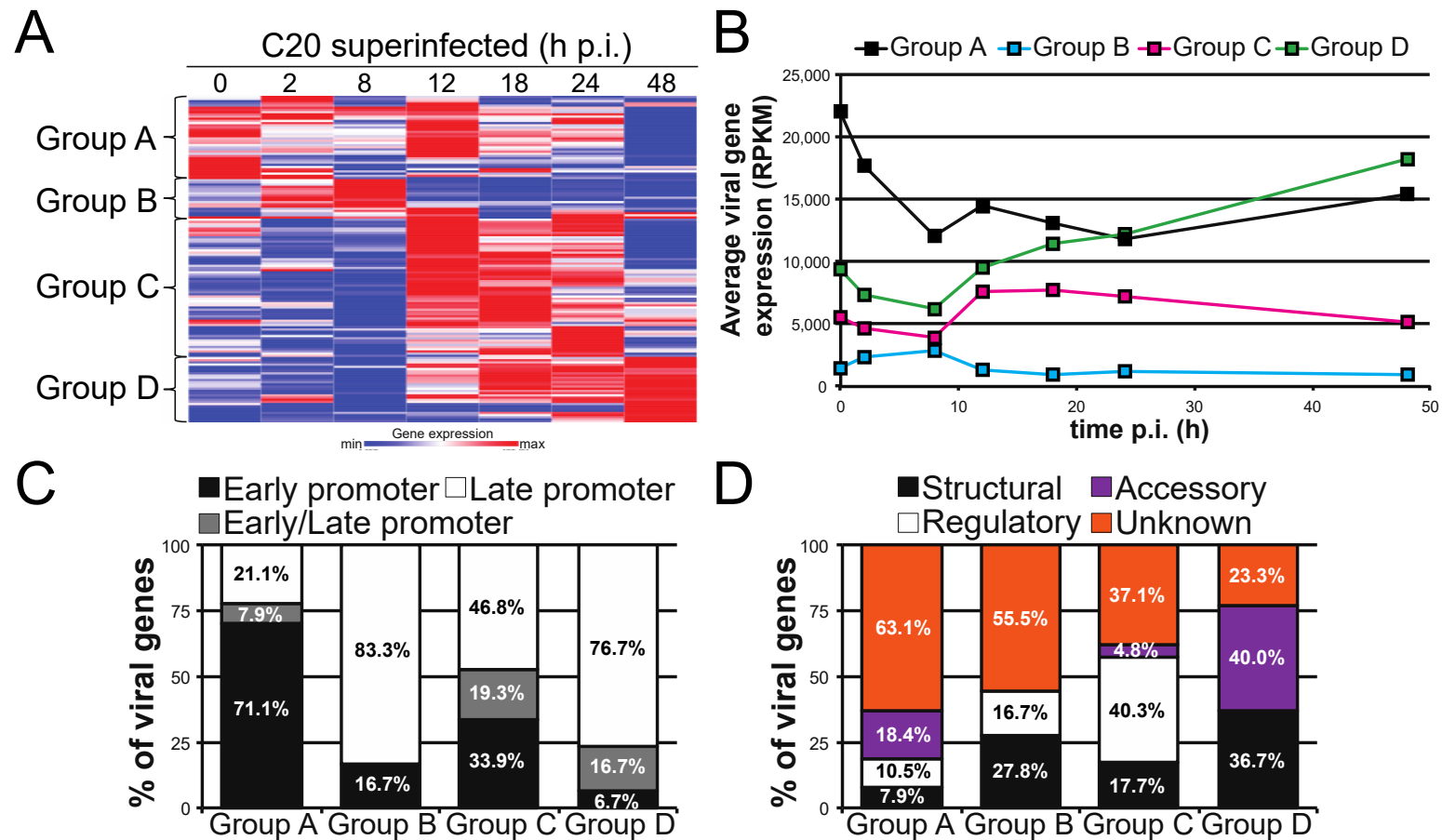


Figure 6.9 Cluster analysis of AcMNPV gene expression pattern in superinfected C20 cells

A) Schematics of normalised gene expression (RPKM) of superinfected C20 cells from 0 to 48 h p.i. Based on their gene expression pattern, viral gene expression was clustered by complete-linkage clustering into three groups: Group A (n=38), Group B (n=18), Group C (n=61) and Group D (n=30). B) For each group, the average gene expression pattern as normalised gene expression levels are shown. C-D) Percentage of viral genes corresponding to C) function and D) promoter motifs are shown. Heat map was visualised using Morpheus Software.

In a productive AcMNPV infection, viral transcripts increased dramatically from 12 h p.i. and presented ~95% of total transcripts at 48 h p.i. (Fig. 6.4). The abundance of virus mRNA resembled therefore the kinetic profile of virus DNA replication, which starts ~5 h p.i., reached a maximum at 18 h p.i. and decreased thereafter (Tjia *et al.*, 1979).

Expression of viral late genes is associated with a change from the host cell RNA polymerase to the virus-encoded α -amanitin resistant polymerase, which recognises the unique TAAG promoter sequence (Fuchs *et al.*, 1983; Rankin *et al.*, 1988; Guarino *et al.*, 1998). Transcription of late genes reached a maximum at 18 h p.i. (Fig. 6.7B) and encoded mainly for structural proteins required for virus spreading (Fig. 6.7C). The most abundant mRNA from 12-24 h p.i. was *p6.9*, which encodes for a structural and arginine rich DNA-binding protein (Table 6.3; Tweeten *et al.*, 1980; Wilson *et al.*, 1987). Other highly abundant mRNAs include the viral transcripts *orf76*, *orf75*, *orf58*, *orf73* and *orf74*. Their function has not been completely resolved yet (Shen *et al.*, 2009; Guo *et al.*, 2010; Hu *et al.*, 2010; Ono *et al.*, 2012; Shen and Chen, 2012). At 48 h p.i. the very late gene *polh* exceeded all transcripts and presented 40% of virus mRNAs (represented 38% of total transcripts). Surprisingly, the second very late gene *p10* only made up 2% of the viral mRNA at 48 h p.i., the last time point p.i. analysed (1.9% of total mRNA; Table 6.3). Moreover, viral very late gene expression did not reach a transcription maximum. Ideally, a later time point p.i. should have been included (e.g. 72 h p.i.). It can be suggested that very late gene expression would have increased even further and even higher than late gene expression at 18 h p.i. (Fig. 6.7B).

Overall, the RNA-seq data from AcMNPV-infected *T. ni* Hi5 cells could for the very first time verify and validate the baculovirus gene expression model to describe the stages and levels of virus gene transcription (Fig. 6.1, 6.7). The major difference between the predicted and experimental validated model was the very late phase of gene expression, which did not reach a maximum of gene expression in the current study. Moreover, viral very late gene expression did not exceed late gene expression levels as it was assumed (Fig. 6.1, 6.7). However, virus gene predictions were made in *Sf* cells maintained in different cell culture media using different AcMNPV wt strains (Kelly and Lescott, 1981; Friesen and Miller, 1985). All those factors may influence virus gene expression.

On the contrary, only ~1.5-2% of the RNA-seq reads could be mapped to the AcMNPV genome in the C20 persistently infected cell line. Moreover, reads were mapped to all virus genes indicating the continuous transcription at a very low level (Fig. S 6.1-6.15, supplementary CD Table S 1, S 2). Therefore, the presence of a persistent virus could be verified, which is distinct from a latent virus infection. So far, only RT-PCR, qPCR or transient expression techniques were employed to verify transcription of some viral genes

in a covert infection either *in vitro* or *in vivo* often using *polh* as a marker (Martínez *et al.*, 2005; Burden *et al.*, 2006; Virto *et al.*, 2013; Graham *et al.*, 2015). Expression of *polh* could be detected by RT-PCR in a cell line derived from *M. brassicae* larvae. Moreover, when reporter plasmids containing a reporter gene either under an early (*ie-1*), late (*p6.9*) or very late (*polh*) promoter were transfected into these cells, the reporter gene was transactivated in every construct although at very low levels. The presence of *polh* mRNA and the transactivation of viral reporter genes indicated that a persistent baculovirus was maintained in this cell line (Hughes *et al.*, 1997). However, the current study confirmed for the first time the continuous expression of all viral genes in an insect cell line harbouring a persistent baculovirus using deep sequencing (Fig. S 6.1-6.15; supplementary CD Table S 6.1, S 6.2).

Virus genes were expressed from both early and later promoters (Fig. 6.7D; Hughes *et al.*, 1997) and a variety of expression patterns were documented in the C20 cell line, which was distinguished from a productive infection (Fig. 6.6-6.8). The C20 cells lacked the typical temporal regulation of virus gene expression (Fig. 6.1, 6.8) and moreover, they did not contain a transition between late and very late phase of expression. Hence, this may be the reason why C20 cells do not die as a cause of the virus infection and moreover, why the virus persists in the cells.

Interestingly, virus genes in C20 cells containing a late promoter motif were expressed highest with *p6.9* transcripts being the most abundant, while immediate early genes were transcribed the lowest throughout the entire time course of the C20 settling time course (Fig. 6.7D; Table 6.3). Other highly abundant transcripts in C20 cells include *orf76*, *Ac-bro*, *v-ubi*, *orf58*, *ctx*, *odv-e27* and the very late gene *polh* (Table 6.3). The essential gene *orf76* encodes for a protein required for BV and ODV envelopment and intranuclear vesicle formation (Hu *et al.*, 2010). The *Ac-bro* encodes for a potential DNA-binding protein that might be required for polyhedra production (Zemskov *et al.*, 2000; Bideshi *et al.*, 2003). The *v-ubi* the gene product localises at the BV inner envelope surface and may function during viral replication to protect viral proteins from degradation by the host cell machinery (Guarino *et al.*, 1995; Haas *et al.*, 1996). The genes *orf58* and *ctx* seems to be nonessential during the viral replication cycle (Eldridge *et al.*, 1992; Ono *et al.*, 2012). The *odv-e27* encodes for a structural protein (Braunagel *et al.*, 2003).

Cluster analysis according to the timing and abundance of transcripts identified three groups with the one comprising down regulated viral genes being the largest in unchallenged C20 cells (Fig. 6.8). In general, a trend of decreased gene expression could be documented over the time course of observation (Fig. 6.8; Table 6.4). Of the down regulated genes most of them contained an early promoter motif and encoded for

regulatory proteins. The smallest group (Group 3) contained up regulated genes and encoded for late and structural protein (Fig. 6.8C). The persistent C20 cell line showed similarities to a lytic infection in regard of the reduction of early and regulatory gene transcription and increased expression of late and structural genes over time (Fig. 6.7, 6.8). It should be noted that only three time points of unchallenged C20 cells p.s. were analysed and that the time interval between them was high due to the restriction of samples size during RNA-seq (more accurate: sufficient average raw read numbers per library). Therefore, gene expression changes during the time intervals could not be detected.

However, the way covert infections are regulated and the mechanism for the maintenance of low level of transcription are still unknown although a number of persistent viruses have been described (e.g. baculoviridae, flaviviridae, paramyxoviridae; McIntosh and Ignoffo, 1981; Burden *et al.*, 2003; reviewed by Rima and Duprex, 2005; Mlera *et al.*, 2014). Infection of *Sf* cells with an AcMNPV, in which the *p35* coding sequence was deleted or exchanged with *lacZ* resulted in the establishment of a persistent baculovirus infection (Lee *et al.*, 1998). The protein P35 usually prevents apoptosis in *Sf* cells and counteracts the host cell antiviral defence mechanism (Clem *et al.*, 1991). Two other *inhibitors of apoptosis (iap)* have been described for AcMNPV (Clem and Miller, 1994). Interestingly, the persistent virus could be activated again when either of the viral anti-apoptotic genes was provided by transfection. It was hypothesised that the virus became persistent by a selection of cells, which expressed high apoptotic suppressors (Lee *et al.*, 1998). That viral anti-apoptotic genes may be required for the maintenance of viral persistency was also described for HSV-1, which can cause latent infections in neuronal cells. The *latency-associated transcript (lat)* required for virus reactivation is the only abundantly expressed HSV gene during its latent stage and may have anti-apoptotic activity. When the coding sequence of HSV *lat* was replaced with the unrelated anti-apoptotic gene *iap* from *Cydia pomonella* granulosus virus (CpGV), HSV was reactivated in mice. This further supported the idea that viral anti-apoptotic genes may be required for viral persistence maintenance (Jin *et al.*, 2005). However, baculovirus anti-apoptotic genes were expressed in C20 cells and therefore, the virus-host balance must be preserved independently from either *p35*, *iap-1* or *iap-2* (supplementary Fig. S 6.11) although it is still unknown if the anti-apoptotic genes in C20 are transcribed and mRNA translated.

A phenotype that was observed in C20 cells was its interference with a homologous virus although a sub population of cells become superinfected (Fig. 6.4, 6.5; R. Arinto Garcia and R. D. Possee, personal communication). However, currently it is unknown if there is a variable state of susceptibility in the population or a sub set that was susceptible. Nevertheless, the same phenotype was observed for several persistently-baculovirus

containing cell lines, e.g. in *Sf* cells harbouring SfNPV or AcΔp35. Additionally, it was observed in *T. ni* cells containing the *Heliothis zea* nucleopolydnavirus (HzNV), which previously belonged to the same virus family as a baculovirus (McIntosh and Ignoffo, 1981; Burand *et al.*, 1983; Lee *et al.*, 1998). It was even reported to be a common phenomenon in persistently infected human cells by flaviviruses (reviewed by Salas-Benito and De Nova-Ocampo, 2015) or in baby hamster kidney fibroblast by arenaviruses (Ellenberg *et al.*, 2007). Exclusion of superinfection is thought to be associated with rearrangements of the actin cytoskeleton as AcMNPV-infected *Sf9* cells could get infected with a second baculovirus after the inhibition of actin polymerisation by cytochalsin D (Beperet *et al.*, 2014).

Cluster analysis of viral mRNA over the time course of infection of C20 cell challenged by a homologous virus identified four distinct groups. Three groups (Group 2-4) followed a similar expression pattern as viral genes in a productive infection (Fig. 6.9) with a maximum reached either at 8 h p.i, 12-18 h p.i or >48 h p.i., respectively. Group 1 mRNAs were transcribed in a unique pattern that could not be observed in an overt infection (Fig. 6.7B, 6.9). Expression of those genes decreased over the time course of superinfection. Interestingly, genes in this cluster mainly contained an early promoter motif and encode for proteins with unknown functions. It may be speculated that genes with an unknown function may be required for the maintenance of a persistent baculovirus infection.

Alterations of phenotypes in persistently *in vitro* cultures have been observed frequently also related to virus interference. For example, persistently infected *Sf9* cells by the arbovirus St Louis encephalitis (SLE) could be superinfected with a baculovirus (Zhang *et al.*, 1993).

Moreover, covert infections are often associated with an increased presence of defective interfering particles (DIF; reviewed by Huang and Baltimore, 1970). For example, virus that was recovered from *T. ni* cells containing a persistently infected HzNV contained a high percentage of DIF (Burand *et al.*, 1983). In addition, ts mutations inside the viral genome were linked to the establishment and/or maintenance of virus persistency (reviewed by Preble and Youngner, 1975). Characterisation of mosquito cell cultures harbouring a long-term persistent SLE revealed different phenotypes in respect to plaque size, temperature sensibility and relative amount of viral protein produced. Hence, alterations of phenotypes of persistently infected cell lines are dependent on the cell type highlighting the relevance of the host cell in the evolution of the virus (Randolph and Hardy, 1988). As the persistent virus collected from C20 is infectious in insect cells (R. D. Possee, personal communication), the possibility of high percentages of DIF and ts

mutations could be excluded. However, the virus genome of the C20 cell line must have undergone some form of evolution as indicated by the genome alterations of the persistent virus. Particularly interesting would be how the evolution of the cell line might influence or modulate specific cellular pathways. For example, how the host cell apoptotic pathway is affected by the covert infection, if transcriptional reduction of virus genes is mediated by the modulation of host chromatin or if translation of already transcribed mRNAs are regulated by a RNAi response of the host cell (detailed analysis and discussion in Chapter 7).

However, it should be kept in mind that with Illumina® deep sequencing the entire population of cells was analysed. Although the C20 cell line was established by single cell dilution, it is currently unknown if the cell line contains a mixed population of viruses and how many of the C20 cells are persistently infected. Both could lead to variations in transcription profiles in a single population. A method to answer these questions would be by the performance of single cell RNA-seq. That individual cells may regulate virus and host gene expression differently is indicated but the spontaneous expression and accumulation of polyhedrin as observed occasionally in some cells and moreover, as a subpopulation of C20 cells is accessible for superinfection (R. Arinto Garcia, R. D. Possee, personal communication).

Another interesting aspect is the presence of the alphanodavirus TNCL in both analysed cell lines *T. ni* Hi5 and C20 (Table 6.2). In *T. ni* Hi5 cells TNCL expression is induced by the infection of a baculovirus (Li *et al.*, 2007). It would be interesting to determine how an already persistent virus acts in combination with a second persistent virus, also in the aspect of virus interference. As the TNCL contains a positive-sense RNA genome, which is immediately translated and is therefore not polyadenylated, the mapped reads to its genome were not qualitative (Li *et al.*, 2007). Hence, it is impossible to determine the effect of the persistent virus in C20 cells on TNCL gene expression by RNA-seq. However, these questions could be answered by the performance RT-PCR analysis.

6.8 Conclusion

To gain a specific and detailed picture of viral gene expression in an insect cell line harbouring a persistent baculovirus infection, strand-specific RNA-seq was performed on C20 cells and C20 cells challenged with AcMNPV and compared to its parental *T. ni* Hi5 cell line. First, the temporal regulation of baculovirus gene transcription into the four distinct temporal phases (immediate early, early, late and very late) and their levels of expression were validated in an overt infection. Second, analysis of C20 cells revealed that all AcMNPV genes were expressed at low levels without distinct temporal phases. The availability of an *in vitro* persistent baculovirus infection offers an imperfect model to

begin to understand how the same phenomenon occurs in host insect populations in nature.

Chapter Seven:

Host transcriptome analysis of an insect cell line harbouring a persistent infected baculovirus

Data were taken from Chapter 6. Differential gene expression analysis of the host was performed by K. Bao (BTI). I performed all downstream analyses at Oxford Brookes University.

7.1 Introduction

Baculovirus infections are typically associated with morphological changes of the host including the rearrangement of the actin and microtubule cytoskeleton, cell cycle arrest and the shut down of host gene expression. Moreover, the establishment of an infection and the viral replication cycle absolutely depends on the host cell machinery (Blissard and Rohrmann, 1990; Possee *et al.*, 2010; Rohrmann, 2013).

Internalisation of AcMNPV is mediated by GP64 from the cell surface by an unknown receptor into clathrin-coated pits (Blissard and Wenz, 1992; Hefferon *et al.*, 1999; Long *et al.*, 2006). After scission of the clathrin-coated vesicles from the plasma membrane, the clathrin coat is removed and the vesicle fuses with an early endosome (reviewed by Robinson, 2015). Furthermore, uptake of AcMNPV is believed to be dependent on the lipid composition of the plasma membrane (Chernomordik *et al.*, 1995; Tani *et al.*, 2001). The plasma membrane lipid bilayer is mainly composed of sterols, sphingolipids and phospholipids. Membrane modifications alter its fluidity and affect their function including endocytosis (reviewed by Holthuis and Menon, 2014).

The endosomal sorting complex required for transport (ESCRT) is part of the endosomal trafficking system and plays crucial roles in the biogenesis of multivesicular bodies but also in cell division by mediating membrane remodelling and budding. It consists of five protein complexes ESCRT0-ESCRTIII and Vps4-Vta and several associated proteins (reviewed by Hurley, 2015). This pathway has been adopted by AcMNPV for both virus entry and nuclear virion egress (Li and Blissard, 2012; Yue *et al.*, 2018). Another highly conserved group of cellular proteins is the soluble N-ethylmaleimide-sensitive factor receptors (SNARE) proteins, which have been implicated to play a role at various steps (virus entry and egress) in the AcMNPV replication cycle (Guo *et al.*, 2017). This group of proteins controls the fusion of cellular membranes, e.g. during intracellular trafficking, signal transduction or endocytosis (reviewed by Han *et al.*, 2017). Following acidification of the endosomes, the virion envelope fuses with the endosome membrane and the nucleocapsid is released into the cytoplasm (Granados and Lawler, 1981).

After the anterograde transport of the virion to the nucleus, it traverses the nuclear membrane via the nuclear pore complex (NPC). The virion is subsequently uncoated inside the nucleus and replication occurs. The progeny virions may egress via the nuclear pore complexes (Goley *et al.*, 2006; Au and Panté, 2012). Although other nucleocapsid egress pathways have been implicated (e.g. by budding; Hess and Falcon, 1978; Fraser and Hink, 1982; Williams and Faulkner, 1997). Nuclear entry and the potential exit via NPC were associated with transcriptional changes of the nucleoporins (nups) and other genes contributing to the NPC (Chen *et al.*, 2014). Moreover, transcriptional regulation

and translation may be controlled by remodelling of the host chromatin by histone deacetylases and by the mRNA export pathway (Chen *et al.*, 2014).

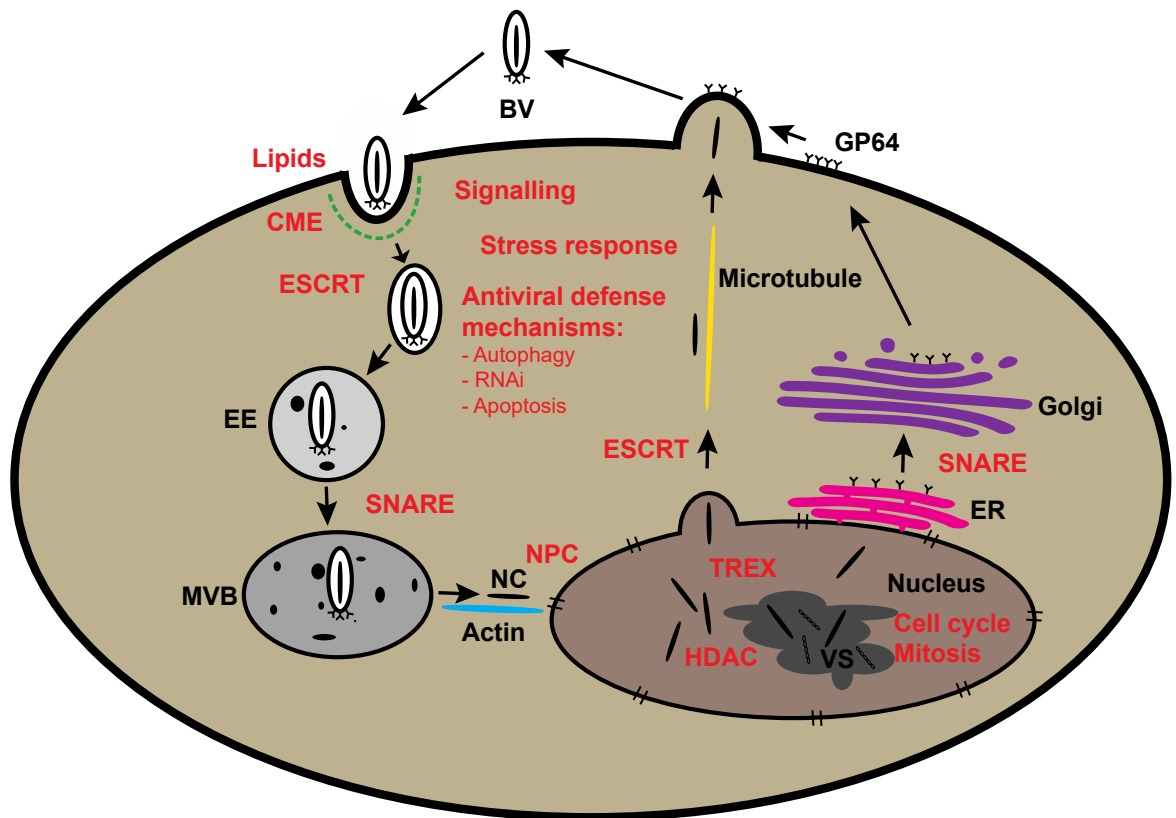


Figure 7.1 Host pathways involved in AcMNPV infection

AcMNPV enters the cell via clathrin-mediated endocytosis (CME), whereby glycoprotein 64 (GP64)-mediated entry is dependent on the plasma membrane lipid composition. Virion trafficking through the endosomal compartments relies on the endosomal sorting complex required for transport (ESCRT) machinery and the soluble N-ethylmaleimide-sensitive factor receptors (SNARE). After endosomal acidification and maturing into a multivesicular body (MVB), the virion fuses with the endosome and thereby releases the nucleocapsids, which traffic in an actin-dependent manner towards the nucleus. The virion enters the nucleus through the nuclear pore complexes (NPCs), where virus uncoating takes place. Virus replication occurs in an electron-dense space, the virogenic stroma (VS) and may be dependent on histone deacetylases (HDAC) and the transcriptional export complex (TREX). The assembled nucleocapsids either bud out into the cytoplasm or exit the nucleus via the NPCs. Afterwards, the virion traffics towards the plasma membrane by microtubules and buds out of the cell, whereby it obtains its envelope. The place of budding is already enriched in GP64, which traffics to the plasma membrane via the endomembrane system through the endoplasmic reticulum (ER) and the Golgi apparatus, possibly in a SNARE protein dependent manner. Moreover, AcMNPV infection causes a cell cycle arrest, modifies cellular signalling, leads to a stress response of the cell and activates the antiviral defence mechanisms.

Cellular pathways are usually regulated by signal transduction, which can be controlled by the virus (reviewed by Mercer and Helenius, 2009; Chi and Liu, 2013). The main host signalling pathways include the mitogen-activated protein kinase (MAPK), the Janus kinase/signal transducers and activator of transcription (JAK/STAT), the PI3K and nuclear factor kappa B- inhibitor of kappa B (NFkB-IkB) pathways, which regulate a variety of cellular responses and act in a signal transduction network (reviewed by Weitzman and

Yaniv, 1998). The MAPK pathways typically regulate cell growth, response to environmental stress and inflammatory cytokines (reviewed by Pearson *et al.*, 2001; Roskoski, 2012), while JAK/STAT signalling is associated with cell survival, differentiation, the regulation of cell growth and pathogen resistance (reviewed by Rawlings, 2004). The PI3K signalling cascade is required for cell survival, regulation of gene expression, cell metabolism and cytoskeletal rearrangements (reviewed by Cantley, 2002) and the NF κ B-I κ B pathway is involved in gene expression, immunity, inflammation and stress responses (reviewed by Hayden and Ghosh, 2008).

Virus infections also induce stress responses of the cell and may activate different antiviral defence mechanisms (Lyupina *et al.*, 2010, 2011). The most common ones include the degradation of the virions by autophagy, the induction of apoptosis and the degradation of viral mRNA by RNA interference (Tschopp *et al.*, 1998; Ratcliff *et al.*, 1999; Orvedahl and Levine, 2009).

To investigate if and how the evolution of the persistent baculovirus together with its host caused the disruption of virus gene expression (Chapter 6), the host transcriptome responses of C20 cells was examined and compared to AcMNPV-infected *T. ni* Hi5 cell.

7.2 Data analysis

Filtered reads from single-strand RNA-seq (from Chapter 6) were mapped to the *T. ni* genome and RPKM values were calculated (K. Bao; Z. Fei, unpublished results; Fig. 7.2, supplementary CD Fig. S 7.1, S 7.2). Simultaneously, as mapped reads to the AcMNPV genome increased over the time course of virus infection in *T. ni* Hi5 cells (Chapter 6 Fig. 6.4), the number of reads mapped to the host genome was reduced (Fig. 7.2A, C). At the same time, reads mapped to the host genome in unchallenged C20 cells remained constant (>95% of cleaned reads) but they decreased slightly to ~90% if they were superinfected (Fig. 7.2B, C). This would indicate that host genes are transcribed at high levels in the presence of a persistent baculovirus. It also further supported the hypothesis that a subpopulation of C20 cells could be superinfected with AcMNPV.

7.3 Host transcriptome

To investigate how the persistent virus in C20 cells affected the response of the host cells, the expression of host genes at each time point p.s. was compared to mock-infected *T. ni* Hi5 cells (as DGE) and in addition compared to the response of AcMNPV-infected *T. ni* Hi5 cells (Kan Bao; Table 7.1; supplementary CD Table S 7.3). For this, gene expression was divided into two groups, either up- or down regulated, which in turn were further subdivided into a high ($FC \geq 10$ or ≤ 0.1), medium ($FC < 10$ or > 0.1) or low response ($FC < 5$ or > 0.5 ; Table 7.1). To monitor significant changes in either gene up- or

down regulation only RPKM $\geq$ 5 were included (with $p < 0.05$; Chen *et al.*, 2014). A RPKM value of three approximately corresponds to one transcript in mammalian cells (Mortazavi *et al.*, 2008). Host genes with RPKM values lower as five were considered either as repressed (RPKM $\geq$ 5 in control=*T. ni* Hi5 mock but RPKM $<$ 5 in C20 sample, with $p < 0.05$) or as induced (RPKM $<$ 5 in control=*T. ni* Hi5 mock but RPKM $\geq$ 5 in C20 sample, with $p < 0.05$). In unchallenged C20 cells only a small portion of the 14,372 *T. ni* genes were up (1.0% in C20 6 h p.s.) or down (2.6% in C20 6 h p.s.) regulated compared to mock-infected *T. ni* Hi5 cells. A slightly higher proportion of host genes were either induced (1.9% in C20 6 h p.s.) or repressed (2.6% in C20 6 h p.s.; Table 7.1) in unchallenged C20 cells. Overall, the majority of host transcription was not regulated (Table 7.1) indicating that *T. ni* genes were expressed in a persistent infection and were not shut down as in a productive infection (Rankin *et al.*, 1988; Nobiron, 2003; Salem *et al.*, 2011; Chen *et al.*, 2014).

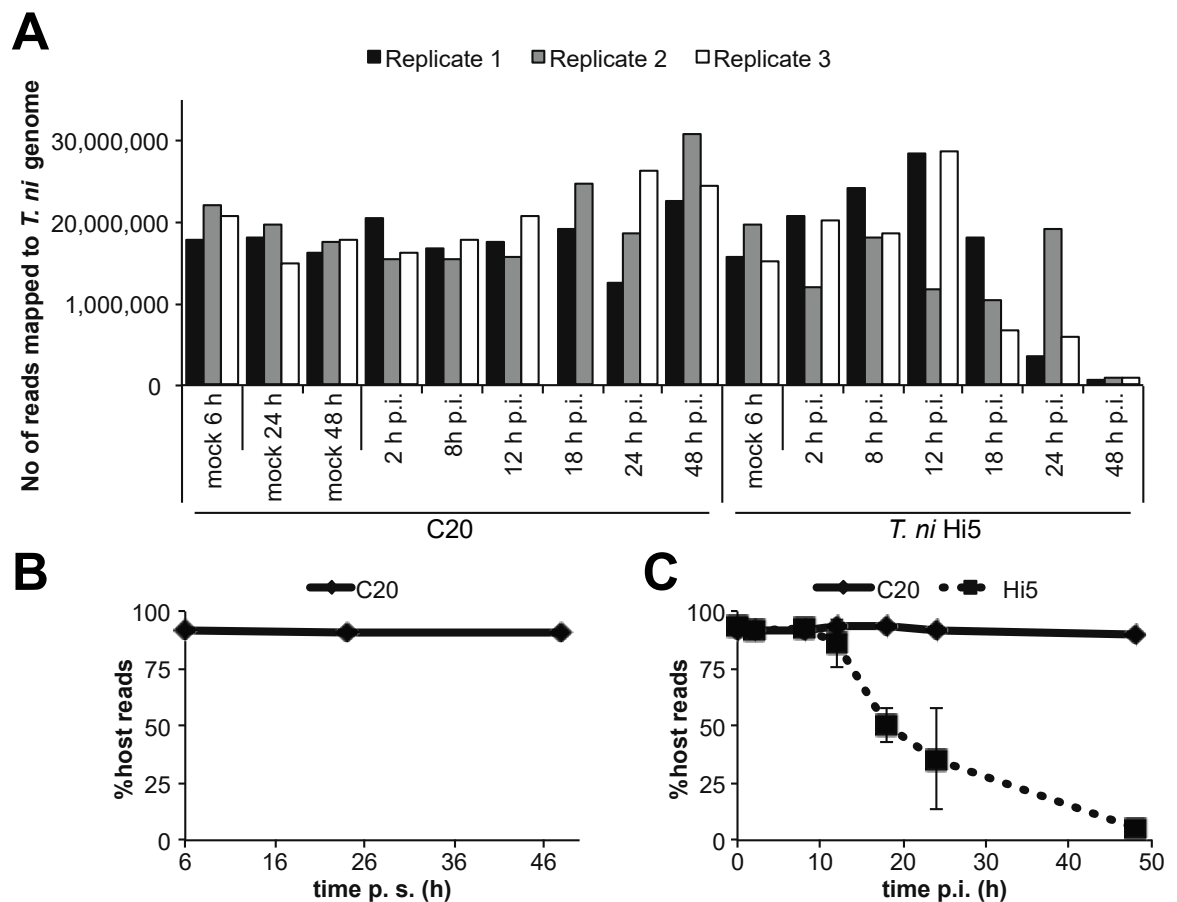


Figure 7.2 Sequencing statistics

Total RNA of C20 cells and *T. ni* Hi5 cells or AcMNPV-challenged C20 or *T. ni* Hi5 cells was extracted at the indicated time points post settling (p. s.) or post infection (p. i.). Strand-specific libraries were generated (Chapter 6) and deep sequencing was performed using Illumina® deep sequencing platform. Total number of Illumina® RNA sequencing reads for each replicate that was mapped to the A) *T. ni* genome. Average percentage of reads in B) unchallenged C20 or C) AcMNPV-infected C20 and *T. ni* Hi5 cells that were mapped to the host genome.

Surprisingly, when the response of the host genes in a productive virus infection was analysed not even 5% of the transcriptome was reduced at 48 h p.i. (Table 7.1). Moreover, no increase in down regulated or repressed genes could be observed over the time course of infection as in previous studies (Chen *et al.*, 2014). The explanation for this might be the high standard deviations in *T. ni* Hi5 samples from 18-48 h p.i. (Fig. 7.2; supplementary CD Table S 7.1, S 7.2) and therefore, the response of the host transcriptome could not be considered as significant.

7.4 Most highly up and down regulated host genes

Subsequently, each of the twenty most highly induced, repressed, up- and down regulated host genes in unchallenged C20 cells were identified by the comparison (DGE analysis) to unchallenged *T. ni* Hi5 cells (Table 7.2-7.5; K. Bao). As standard deviations of virus-infected *T. ni* Hi5 cells were in general high at later time points p.i., only *T. ni* Hi5 mock samples were included for the analysis. Of the most highly regulated host genes only 36.25% could be annotated, which represents the total *T. ni* genome annotation of ~1/3 (Chen *et al.*, 2014; Z. Fei, personal communication). The most highly induced host genes in C20 cells encoded for transmembrane receptors (glutamate-gated channel, trehalose transporter and neural acetylcholine receptor), a RNA polymerase II subunit, α -tubulin and an enzyme involved in the fatty acid biosynthesis (Table 7.2).

Interestingly, five out of the twenty most highly up regulated host genes in C20 cells encoded for GST, which is a detoxification enzyme but also a cell signalling modulator in response to oxidative stress (Table 7.3; reviewed by Sheehan *et al.*, 2001). Another highly up regulated gene in C20 cells was *hsp70* (Table 7.3), which also encodes for a protein involved in the protection of oxidative stress (reviewed by Fernández-Fernández *et al.*, 2017). Other most highly up regulated genes included an *UDP-glycosyltransferase*, *rab37* encoding for a key protein in autophagosome formation (Table 7.3; Sheng *et al.*, 2017; Song *et al.*, 2018) and a peptidoglycan recognition protein (Table 7.3).

The most highly repressed host genes in C20 cells encoded for proteins involved in endocytosis and uptake of proteins (caveolin, inorganic phosphate transporter), ligands in cellular signalling (jagged protein) and enzymes involved in the synthesis of spingolipids (ceramide phosphoethanolamine synthetase; Table 7.4; reviewed by Holthuis and Menon, 2014).

Table 7.1 Pairwise DGE analysis of host genes compared to *T. ni* Hi5 mock cells

Sample	Induced	Fold Change						Repressed	Total regulated	%Up*	%Down*	%Regulated	%Not regulated
		Up regulated			Down regulated								
		≥10	<10-5	<5-2	>0.2-0.5	>0.1-0.2	≤0.1						
C20 unchallenged 6 h	271	7	15	126	287	41	11	403	1161	2.92	5.16	8.08	91.92
C20 unchallenged 24 h	384	4	15	160	908	47	11	564	2093	3.92	10.65	14.56	85.44
C20 unchallenged 48 h	429	5	22	261	638	29	8	471	1863	9.69	7.97	17.66	82.34
<i>T. ni</i> Hi5 2 h p.i.	0	0	0	3	2	1	0	14	20	0.02	0.12	0.14	99.86
<i>T. ni</i> Hi5 8 h p.i.	259	0	1	103	234	3	0	254	600	2.53	3.42	5.94	94.06
<i>T. ni</i> Hi5 12 h p.i.	45	1	1	15	69	7	0	65	203	0.43	0.98	1.41	98.59
<i>T. ni</i> Hi5 18 h p.i.	176	1	1	158	341	12	0	181	870	2.34	3.72	6.05	93.95
<i>T. ni</i> Hi5 24 h p.i.	85	0	3	72	254	4	0	74	492	1.11	2.31	3.42	96.58
<i>T. ni</i> Hi5 48 h p.i.	232	11	37	31	631	18	1	42	1003	2.16	4.81	6.98	93.02

*% Up and down regulated gene expression include induced and repressed genes, respectively; Abbreviation: DGE: differentially expressed genes

Table 7.2 Most highly induced *T. ni* genes in C20 cells⁺

most highly induced <i>T. ni</i> genes in C20 mock with <5 RPKM					
Gene ID	Annotation	RPKM		FC	adjust.p
		<i>T. ni</i> Hi5 mock	C20 mock 6 h		
Tni13493*		0	23.12	2312	0.000406978
Tni05060*		0	22.46	2246	5.41E-29
Tni20555*		0.01	17.85	1338.75	4.12E-36
Tni03409**		0	9.04	903.67	2.51E-10
Tni00628*		0.01	9.46	709.5	4.13E-38
Tni01753*	dna-directed rna polymerase ii subunit rpb1	0.03	17.27	575.56	2.83E-34
Tni13268*	tubulin alpha chain-like	0.03	14.66	488.78	2.21E-11
Tni08511*	glutamate-gated chloride channel isoform x11	0.05	20.91	448	2.81E-27
Tni10389*	facilitated trehalose transporter tret1-like	0.08	19.72	257.22	2.65E-39
Tni14077*		0.14	32.73	239.46	4.54E-16
Tni06910***	uncharacterised protein part	0.03	5.53	207.38	0.002700063
Tni02755*		0.06	11.3	188.33	5.52E-41
Tni11318*		0.72	132.66	183.41	1.39E-55
Tni00203*	acyl-desaturase-like	0.04	7.26	181.58	6.25E-38
Tni12174**	neuronal acetylcholine receptor subunit alpha-7	0.06	9.59	169.18	2.25E-35
Tni08268**		0.55	87.37	158.85	3.22E-09
Tni00203*	acyl-desaturase-like	0.04	7.26	181.58	6.25E-38
Tni12174**	neuronal acetylcholine receptor subunit alpha-7	0.06	9.59	169.18	2.25E-35
Tni08268*		0.55	87.37	158.85	3.22E-09

+RPKM and fold change (FC) value are shown for C20 6 h p.s., *one of the most twenty induced host gene in all three C20 samples (6 h p.s., 24 h p.s., 48 h p.s.), **one of the most twenty induced host gene in C20 6 h p.s. and a second time point p.s., ***one of the most twenty induced host gene in C20 6 h p.s. only.

The most highly down regulated host genes in C20 cells comprised genes encoding for proteins required for *in vivo* development (larval pupal rigid cuticle protein, nidogen and shootin-1). In addition, transcription of the immune-related gene *cobatoxin* and *moesin ezrin radixin*, the gene product of which regulate cytoskeleton remodelling were reduced (Table 7.5; Selisko *et al.*, 1998; reviewed by Neisch and Fehon, 2011).

7.5 Cellular pathway analysis in C20 cells

For the invasion of cells and the establishment of an infection as well as spreading, the virus makes use of specific cellular pathways (Rohrmann, 2013). Therefore, to investigate the reasons for the C20 cell phenotype (production of low titre of virus and the exclusion of superinfection) in more detail, various host-associated pathways were examined more closely (supplementary CD Table S 7.4).

Table 7.3 Most highly up regulated *T. ni* genes in C20 cells*

most highly up regulated <i>T. ni</i> genes in C20 cells mock with ≥ 5 RPKM					
GeneID	Annotation	RPKM		FC	adjust.p
		<i>T. ni</i> Hi5 mock	C20 mock 6 h		
Tni10466*		6.56	330.95	50.48	1.17E-25
Tni05898*	glutathione s-transferase	6.3	153.29	24.33	3.73E-14
Tni13066*	glutathione s-transferase like	10.79	202.04	18.73	5.34E-24
Tni12034*	udp-glycosyltransferase utg3	9.07	99.85	11.01	1.78E-20
Tni11381*		8.44	91.55	10.84	2.73E-17
Tni13956*		13.89	150.59	10.84	2.25E-21
Tni13067*	glutathione s-transferase	96.49	1035.2	10.73	2.25E-21
Tni12637**		82.33	785.59	9.54	7.23E-05
Tni04958*		5.33	45.24	8.48	2.21E-04
Tni20072**		19.86	144.21	7.26	3.06E-06
Tni05252***		12.2	83.32	6.83	1.94E-04
Tni13064**		6.6	43.97	6.67	2.88E-13
Tni10440**	heat shock protein 70	18.44	112.96	6.13	5.10E-06
Tni04707***	ras-related protein rab-37 isoform x1	11.3	68.86	6.1	0.00017
Tni09315*		13.4	81.52	6.08	3.77E-05
Tni20088**		16.65	99.01	5.95	1.15E-07
Tni12036*	glutathione s-transferase-like	10.26	58.61	5.71	4.97E-08
Tni02319***		7.05	39.88	5.66	3.48E-11
Tni00812***	peptidoglycan-recognition protein lb-like	35.2	194.64	5.53	4.45E-07
Tni20278*	microsomal glutathione s-transferase 1-like	53.5	290.9	5.44	8.02E-10

+RPKM and fold change (FC) value are shown for C20 6 h p.s., *one of the most twenty up regulated host gene in all three C20 samples (6 h p.s., 24 h p.s., 48 h p.s.), **one of the most up regulated induced host gene in C20 6 h p.s. and a second time point p.s., ***one of the most twenty up regulated host gene in C20 6 h p.s. only.

The *T. ni* unigenes of specific cellular pathways were adapted from Chen *et al.*, 2013. Functional genes were identified by Basic Local Alignment Search Tool using a nucleotide search query (Blastn) against the assembled *T. ni* genome (Z. Fei, personal communication). The annotation of the adapted pathways were performed in collaboration with A. Shrestha (BTI; supplementary CD Table S 7.4).

Additionally, protein groups of specific conserved cellular pathways (lipid synthesis, cell cycle progression, mitosis and autophagy) were collected from *B. mori* (Silkworm Knowledgebase- SilkDB), *Drosophila melanogaster* (FlyBase), *S. cerevisiae* (Saccharomyces Genome Database- SGD) and *Homo sapiens* (NCBI) databases and identified by translated nucleotide databases using a protein query (tBlastn) with E-values

of $<1e^{-10}$ and alignments of $>25\%$ (supplementary CD Table S 7.4; data from *B. mori*, *D. melanogaster*, *S. cerevisiae* and *H. sapiens* not shown; Zhang *et al.*, 2009).

Table 7.4 Most twenty highly repressed *T. ni* Hi5 genes in C20 cells*

most highly repressed <i>T. ni</i> genes in C20 mock with <5 RPKM					
Gene ID	Annotation	RPKM		FC	adjust.p
		<i>T. ni</i> Hi5 mock	C20 mock 6 h		
Tni03295*		6.46	0.01	0	2.05E-23
Tni05540*	caveolin-3-like isoform	27.85	0.1	0	2.71E-28
Tni05617*		10.04	0	0	4.58E-25
Tni05651*		13.27	0.03	0	2.22E-50
Tni06078*		22.76	0.04	0	3.04E-24
Tni09436*		18.46	0	0	1.04E-38
Tni11004*		10.8	0.04	0	5.87E-26
Tni20032*		10.53	0.04	0	1.53E-21
Tni00653*		10.08	0.05	0.01	1.17E-25
Tni02849***		6.96	0.1	0.01	3.00E-21
Tni03453*		17.33	0.14	0.01	5.41E-15
Tni06079*		117.33	0.89	0.01	1.03E-42
Tni06550*		5.03	0.04	0.01	5.51E-15
Tni08752*		16.47	0.15	0.01	4.09E-31
Tni13144*	protein jagged	5.27	0.05	0.01	2.07E-20
Tni03483***		71.25	1.69	0.02	7.68E-26
Tni04037***	inorganic phosphate cotransporter	15.73	0.32	0.02	3.58E-22
Tni05827**		5.19	0.09	0.02	8.44E-06
Tni06195*		5.99	0.13	0.02	2.88E-18
Tni09929***	ceramide phosphoethanolamine synthase	13.92	0.26	0.02	7.14E-18

+RPKM and fold change (FC) value are shown for C20 6 h p.s., *one of the most twenty repressed host gene in all three C20 samples (6 h p.s., 24 h p.s., 48 h p.s.), **one of the most repressed induced host gene in C20 6 h p.s. and a second time point p.s., ***one of the most twenty repressed host gene in C20 6 h p.s. only.

Although gene expression values of all three unchallenged C20 samples are shown in Table S 7.4, the analysis was narrowed to unchallenged C20 6 h p.s., which was in addition considered as time point 0 h p.i., as already discussed in Chapter 6. As it was hypothesised that gene expression variations exist between single C20 cells (Chapter 6), the significance of host gene expression during the analysis process was not considered in more detail although it was calculated (supplementary CD Table S 7.3). Furthermore, although expression of various cellular pathway-related genes were analysed (Figure 7.3-7.20; supplementary CD Table S 7.3), the following paragraphs only point out some highlights and the main differences determined between the C20 and its parental cell line. This may help to understand why a persistent baculovirus infection is maintained and why the analysed C20 cell line shows some specific phenotypes (e.g. longevity or mainly not susceptible for superinfection).

Table 7.5 Most highly down regulated *T. ni* genes in C20 cells⁺

most highly down regulated <i>T. ni</i> genes in C20 cells mock with >5 RPKM					
GeneID	Annotation	RPKM		FC	adjust.p
		<i>T. ni</i> Hi5 mock	C20 mock 6 h		
Tni20592*	larval pupal rigid cuticle protein 66-like	360.31	8.64	0.02	3.12E-16
Tni06859*	nidogen-2 isoform x4	179.89	7.62	0.04	2.50E-21
Tni07070***	cobatoxin-like protein	90.62	5.58	0.06	5.31E-15
Tni01142**		86.46	6.91	0.08	1.12E-12
Tni05987*		101.04	8.19	0.08	3.55E-13
Tni06488*		89.21	7.52	0.08	3.89E-13
Tni07419***		255.46	20.12	0.08	7.03E-13
Tni02719***		57.89	5.29	0.09	1.78E-11
Tni05505*		161.33	14	0.09	8.59E-12
Tni07692**		152.8	14.72	0.1	4.30E-11
Tni08348**	purine nucleoside phosphorylase-like isoform x1	49.1	5.02	0.1	6.98E-10
Tni02456*		189.89	21.76	0.11	1.06E-09
Tni04741*		85.57	9.71	0.11	2.14E-09
Tni09567*		127.42	14.6	0.11	8.59E-10
Tni03918***	moesin ezrin radixin homolog 1 isoform x2	631.34	79.19	0.13	6.85E-09
Tni10316***	shootin-1	153.44	20.27	0.13	3.88E-08
Tni12767***		60	7.67	0.13	3.20E-08
Tni00399**		78.33	11.08	0.14	1.62E-07
Tni07648***		48.71	6.74	0.14	1.96E-07
Tni00845***		36.61	5.48	0.15	4.67E-06

+RPKM and fold change (FC) value are shown for C20 6 h p.s., *one of the most twenty repressed host gene in all three C20 samples (6 h p.s., 24 h p.s., 48 h p.s.), **one of the most repressed induced host gene in C20 6 h p.s. and a second time point p.s., ***one of the most twenty repressed host gene in C20 6 h p.s. only.

7.5.1 Endocytosis

Genes encoding for proteins required for endocytosis were usually expressed highest between 2-8 h p.i. in AcMNPV-infected *T. ni* Hi5 cells, as expected, while there was a delay of gene expression in superinfected C20 cells. Transcription of endocytosis-associated genes generally peaked after 12 h p.i. in C20 cells (Fig. 7.3). Moreover, genes required for the nucleation of endocytic pits were considered as repressed in C20 cells as they contained RPKM values of less than five (supplementary CD, Table S 7.4) indicating a deficiency of endocytosis in the baculovirus persistently infected cell line.

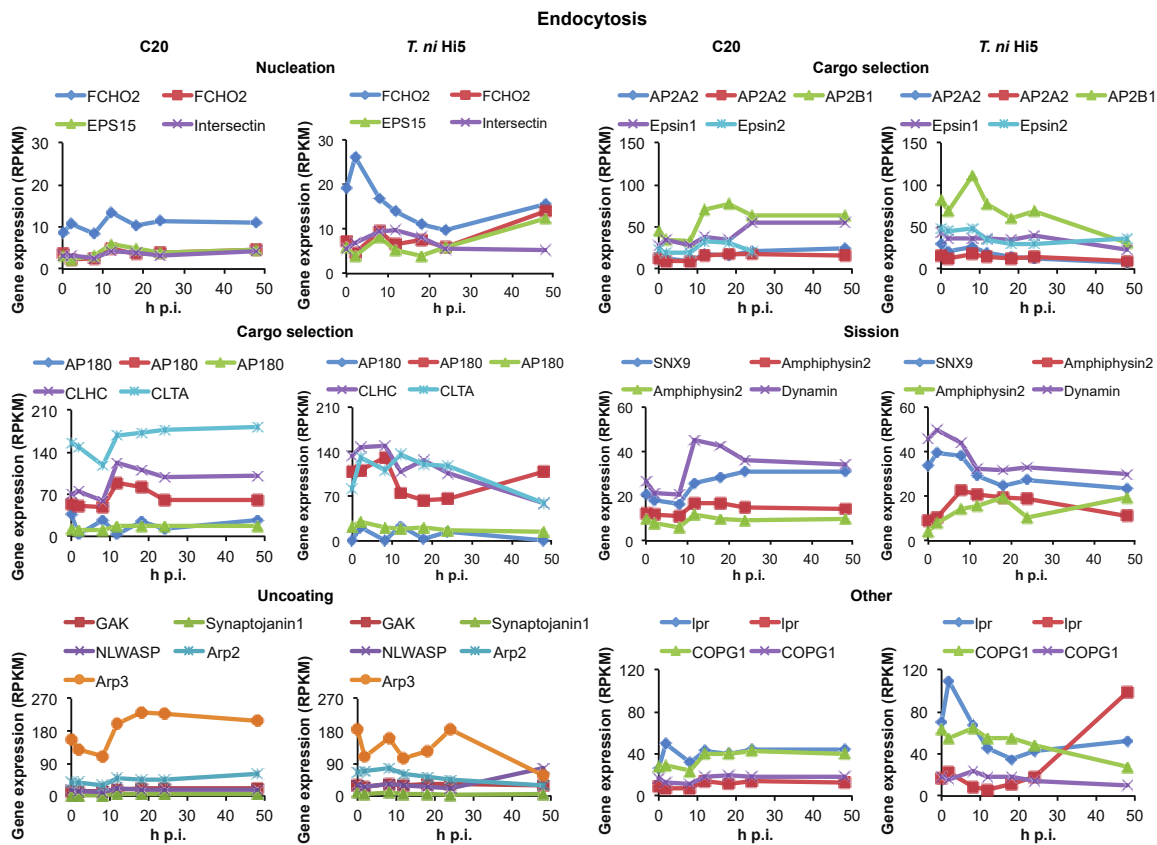


Figure 7.3 Transcription profiles of endocytosis-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: AP: adapter protein, Arp: actin related protein, CLHC: clathrin heavy chain, CLTA: clathrin light chain A, COG1: coatamer protein subunit gamma 1, EPS: epidermal growth factor receptor, FCHO: FCH domain only, GAK: cycline G associated protein, Ipr: isoproterenol, SNX: sorting nexin, NLWASP: neuronal Wiskott-Aldrich Syndrome protein

7.5.2 Lipid Synthesis

The stearoyl-CoA desaturase (SCD) is a key enzyme in the formation of monosaturated fatty acids synthesis and it uses diverse lipids including phospholipids and triglycerids as substrates (reviewed by Paton and Ntambi, 2009). Expression of *scd* in AcMNPV-infected *T. ni* Hi5 increased modestly until 18-24 h p.i. and subsequently declined. On the contrary, *scd* transcription of superinfected C20 cells decreased until 8 h p.i., after which it increased back to its basal level from 0 h p.i. (Fig. 7.4). In addition, genes encoding for the following proteins required for glyceride synthesis decreased in virus-infected *T. ni* Hi5 cell over time: Diacylglycerol Acyltransferase (DGAT), Glycerol-3-Phosphate (GPAT) and Acyl-CoA Synthetase (ACS), while the expression levels of those genes stayed constant in AcMNPV challenged C20 cells (Fig. 7.4). This may suggest that the regulation of lipid synthesis in C20 cell was regulated differently than in *T. ni* Hi5 cells.

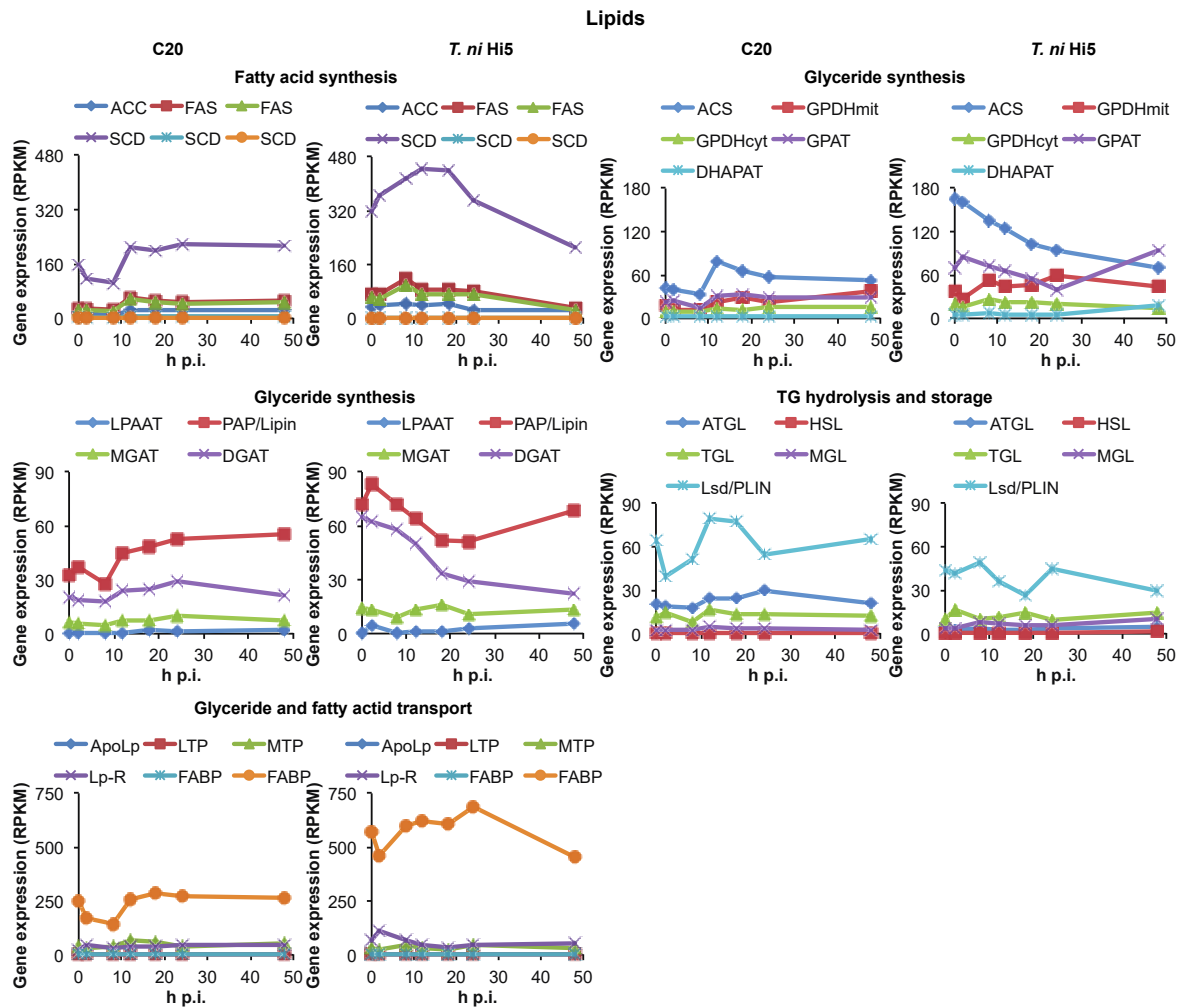


Figure 7.4 Transcription profiles of genes involved in lipid synthesis

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: ACC: Acetyl CoA carboxylase, ACS: Acyl-CoA Synthetase, ATGL: adipose triglyceride lipase, Apolp: apolipoprotein, DHADPAT: dihydroxylacetone phosphate, FABP: fatty acid binding protein, FAS: fatty acid synthase, GPAT: glycerol-3-phosphate phosphate acyltransferase, GPDHcyt: glycerol-3-phosphate DH (cyt), GPDHmit: glycerol-3-phosphate DH (mit), HSL: hormone sensitive lipase, LPAAT: lysophosphatidic acid phosphatase, Lp-R: lipoprotein receptor, Lsd/PLIN: lipid storage droplet protein, LTP: lipid transfer particle, MGAT: monoacylglycerol acyltransferase, MGL: monoglyceride lipase, MTP: microsomal triacylglycerol transfer protein, PAP: phosphatidic acid phosphatase, TGL: triglyceride lipase, SCD: acyl-CoA delta-9 desaturase

7.5.3 ESCRT

In general, gene expression of ESCRT components in AcMNPV-infected *T. ni* Hi5 cells reached their maximum between 2-12 h p.i. highlighting their role in both virus entry and exit (Fig. 7.5; supplementary CD Table S 7.4; Chen *et al.*, 2013). However, transcription of some of the ESCRT genes did not peak and expression levels stayed low during the time course of AcMNPV infection in C20 cell. These genes included *vps4*, *chmp4*, *chmp5*, *chmp6* (all CHMPs are subunits of ESCRT-III), *eap30* (subunit of ESCRT-II) and *vps26* (involved in retrograde transport from ER to the Golgi network; Fig. 7.5; supplementary

CD Table S 7.4; reviewed by Hurley, 2015) suggesting that gene expression of ESCRT-associated components was affected in C20 cells.

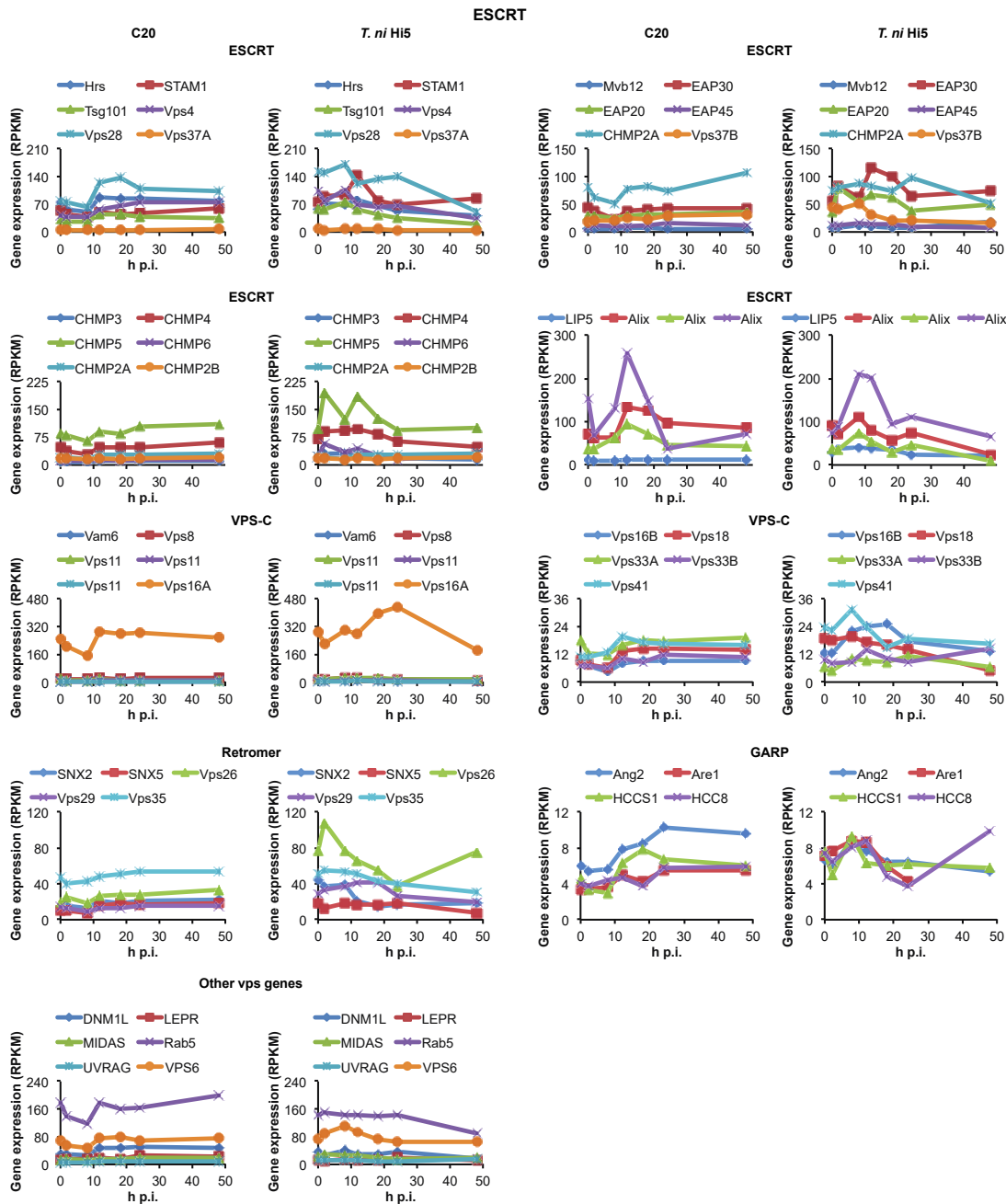


Figure 7.5 Transcription profiles of ESCRT-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Alix: ALG-2 interacting protein X, Ang2: another new gene 2, Are1: a region expressed 1, CHMP: Charged multivesicular body protein, DNM1L: dynamin 1-like protein, EAP: ELL-associated protein, ESCRT: the endosomal sorting complexes required for transport, HCC8: hepatocellular carcinoma protein 8, HCCS1: hepatocellular carcinoma suppressor 1, Hrs: hepatocyte growth factor regulated tyrosine kinase substrate, LEPR: leptin receptor gene related protein, LIP5: LYST interacting protein 5, MIDAS: mitochondrial DNA absence sensitive factor, Mvb12: Multivesicular body sorting factor 12, PI3K: phosphatidylinositol-3-kinase, Rab: Ras-associate protein STAM: signal transducing adaptor protein, SNX: sorting nexin, Tsg101: Tumor susceptibility gene 101 protein, UVRAG: UV radiation resistance associated gene, Vam6: vacuolar morphogenesis protein 6, VPS: vacuolar protein sorting protein

7.5.4 SNARE proteins

The SNARE gene *syntaxin 7* (*Syx7*) encodes for a protein that is part of the core machinery for endocytic vesicle trafficking to the late endosomes (Nakamura *et al.*, 2000). In AcMNPV-infected *T. ni* Hi5 cells, expression of *Syx7* increased until 8 h p.i., and subsequently decreased. On the contrary, transcription of *Syx7* in superinfected C20 declined and reached a minimum at 8 h p.i. (Fig. 7.6). Moreover, transcription of *N-ethylmaleimide sensitive fusion protein* (*NSF*) was reduced (by FC=0.42) in unchallenged C20 cells (6 h p.s.), when compared to mock-infected *T. ni* Hi5 cells (Fig. 7.6; supplementary CD Table S 7.3, 7.4). The NSF gene product is required for the disassembly and process of post-fusion recycling of the SNARE complex (reviewed by Bombardier and Munson, 2015). Another protein that was implicated in the disassembly and recycling of the SNARE proteins but also in other multiple membrane trafficking steps such as membrane docking and fusion to the plasma membrane is SNAP-29 (Su *et al.*, 2001). Transcription of *SNAP-29* decreased after superinfection in C20 cell, whereas it reached a maximum in AcMNPV-infected *T. ni* Hi5 cells at 12 h p.i. (Fig. 7.6). This suggested that the regulation of multiple trafficking steps in C20 alters compared to *T. ni* Hi5 cells.

7.5.5 Nuclear Pore complex

Expression of *Nup155*, which is part of the inner-ring NPC complex, generally declined after AcMNPV infection in *T. ni* Hi5 cells (Fig. 7.7). Transcription of *Nup155* in uninfected C20 cells was reduced (by 0.31-fold) compared to unchallenged *T. ni* Hi5 cells and only slightly decreased from 12 h p.i. after superinfection (Fig. 7.7; supplementary CD Table S 7.3, 7.4). Interestingly, *Nup155* interacts with *GLE1*, which is required for the terminal steps of mRNA transport through the NCP (Rayala *et al.*, 2004; reviewed by Knockenhauer and Schwartz, 2016). Expression of *GLE1* showed a similar trend as *Nup155* in both infected C20 and *T. ni* Hi5 cells (Fig. 7.7) indicating that mRNA transport through the NPC varied in C20 cells when compared to its parental cell line.

7.5.6 Histone deacetylase and Sirtuin genes

Expression of the *histone deacetylase 1* (*HDAC1*) was decreased (by 0.56 fold) in uninfected C20 cells when compared to *T. ni* Hi5 mock cells (Fig. 7.8; supplementary CD Table S 7.3, 7.4). After superinfection, the expression of *HDAC1* did not change, whereas it increased in AcMNPV-infected *T. ni* Hi5 cells reaching a maximum at 8 h p.i. (Fig. 7.8). Furthermore, transcription of *sirtuin 6* (*sirt6*) that may be involved in the repair of DNA damage (reviewed by Lombard *et al.*, 2008), peaked in AcMNPV-infected *T. ni* Hi5 cells at 12 h p.i., whereas it remained low in superinfected C20 cell (Fig. 7.8).

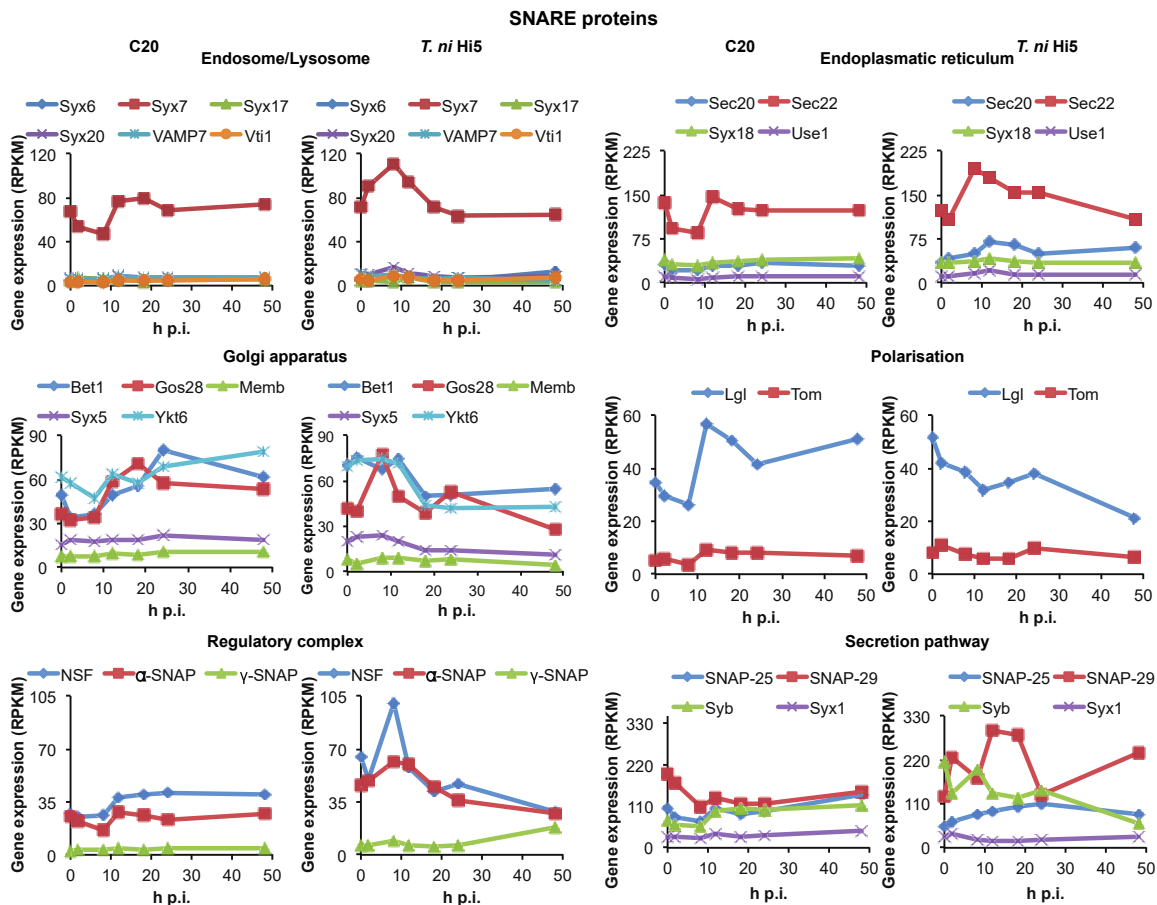


Figure 7.6 Transcription profiles of genes encoding for SNARE proteins

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: α -SNAP: α -soluble NSF attachment protein, Bet1: blocked early in transport, Bos1: Bet1 suppressor 1, Gos: Golgi SNARE protein (size in kDa), Lgl: lethal giant larvae, Memb: mebrin, Myob: myobrevin, NSF: N-ethylmaleimide sensitive fusion protein, Nyv1: new yeast v-SNARE, Pep12: carboxypeptidase Y deficient, Sec: secretory mutant protein, Sed5: suppressor of Erd2 deletion, Sft1: suppressor of Sed5, Slt1: SNARE-like tail-anchored protein 1, Snc1/Snc2: suppressor of the null allele of CAP, SNAP: synaptosome-associated protein (size in kDa), SPO20: sporulation 20, Sro7/Sro77: suppressor of Rho3, Sso1/Sso2: suppressor of Sec1, Syb: synaptobrevin, Syx: syntaxin, Tlg: T-snare affecting a late Golgi compartment, Tom: tomsyn, Ufe1: unknown function essential, Use1: unconventional SNARE in the ER, Vam: vacuolar morphogenesis, VAMP: vesicle-associated membrane protein, Vti1: Vps10 interacting protein, Ykt6: YKL196c-encoding protein

7.5.7 Transcription export pathway

The TREX pathway is required for various mRNA processing steps (e.g. cap formation, splicing events) and is involved in the mRNA export from the nucleus into the cytoplasm (reviewed by Gaouar and Germain, 2013; Heath *et al.*, 2016). Transcription of the *dead box protein 39A/39B* (*DDX39A/DDX39B*), which encodes for a putative RNA helicase and an essential splicing factor (Fleckner *et al.*, 1997), was reduced (by FC=0.39) in C20 cells when compared to *T. ni* Hi5 cells (Fig. 7.9; supplementary CD Table S 7.3, 7.4). Furthermore, expression of the gene *suppressor of transcriptional defects of hpr1 delta by overexpression complex* (*Thoc2*) remained constant over the time course of

superinfection in C20 cells, while its transcription was activated in AcMNPV-infected *T. ni* Hi5 cells and was highest at 2 h p.i. (Fig. 7.9). The Thoc2 is the largest subunit of the TREX-complex required for mRNA biogenesis and acts as an essential scaffold protein (Peña *et al.*, 2012). This may indicate differences in the gene expression processing steps of C20 cells compared to its parental cell line.

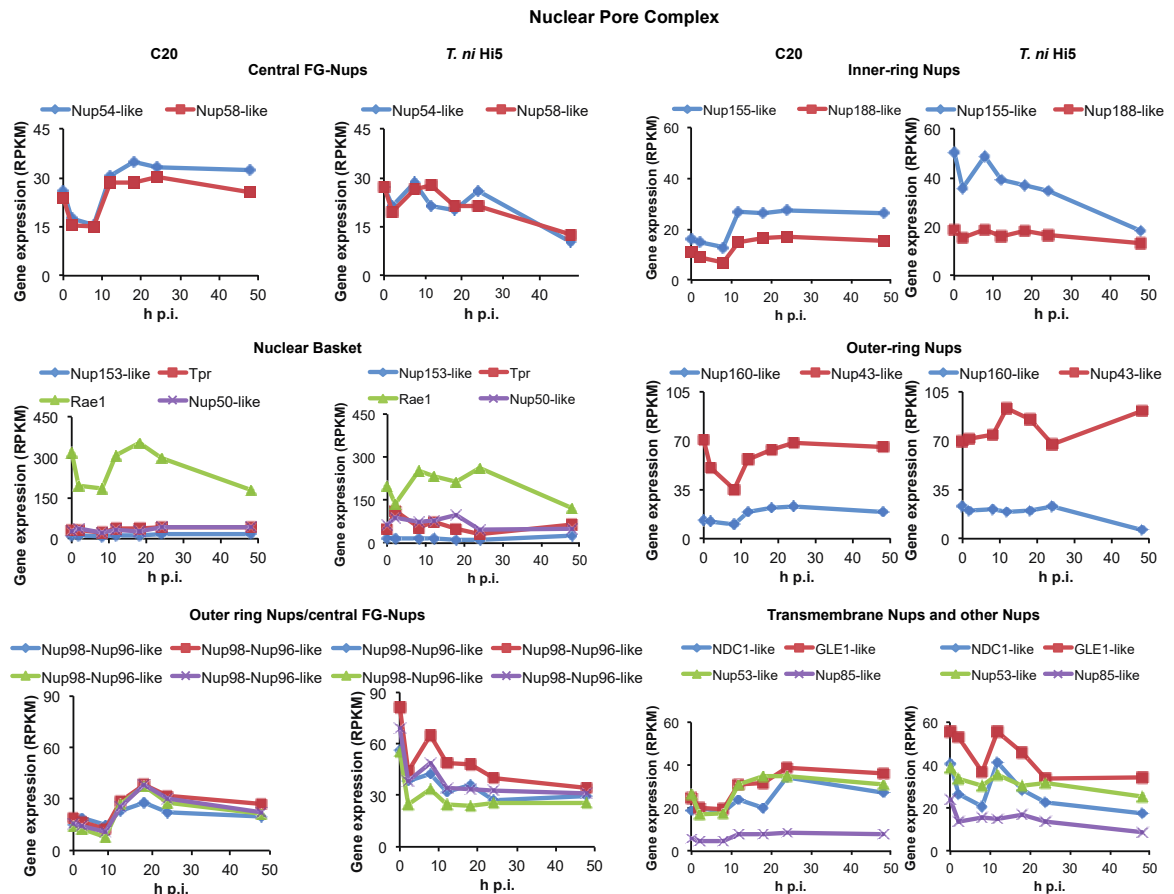


Figure 7.7 Transcription profiles of genes encoding for the Nuclear Pore Complex

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: FG: phenylalanine-glycine repeats, Nup: nucleoporins

7.5.8 Cell cycle progression and mitosis

The division of the cell is highly regulated and involves several checkpoints (points of no return) to ensure proper DNA synthesis, to detect any deficiency in the replication of the genetic material, to recognise any failure during the separation of chromosomes and to inhibit uncontrollable cell division (reviewed by Pines, 2006; Bloom and Cross, 2007; Malumbres, 2011; Carmena *et al.*, 2012; Fisher *et al.*, 2012; Lara-Gonzalez *et al.*, 2012; Bertoli *et al.*, 2013; Sivakumar and Gorbsky, 2015; Gérard and Goldbeter, 2016).

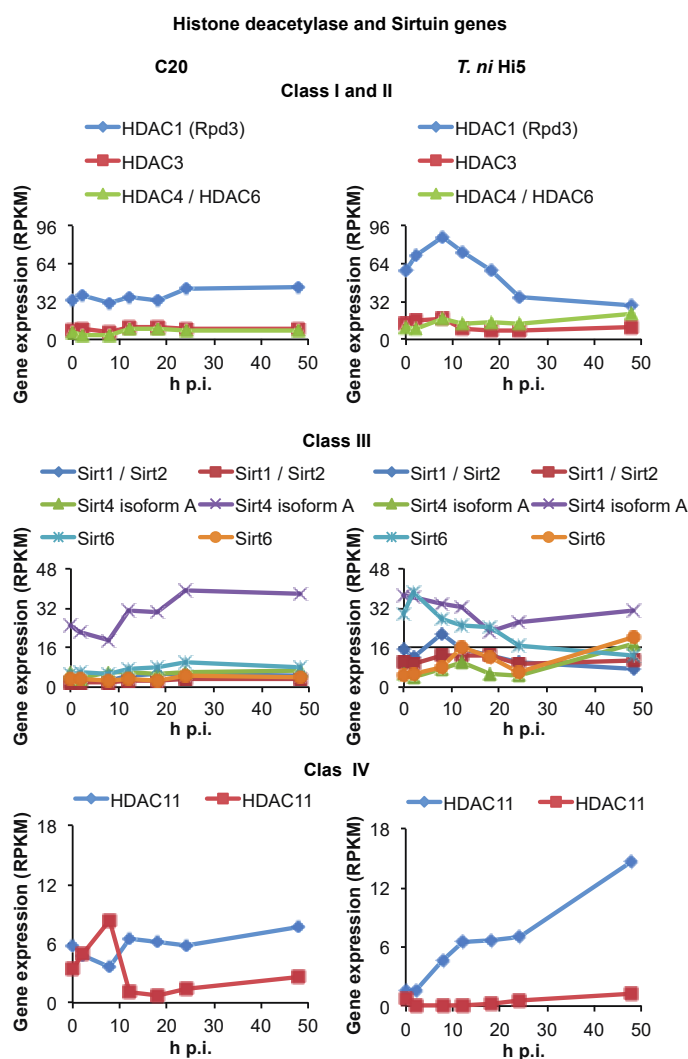


Figure 7.8 Transcription profiles of Histone deacetylase and Sirtuin genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: HDAC: histone deacetylase, Sirt: Sirtuin

One of the master regulators of the cell cycle is the retinoblastoma protein (Rb). It negatively regulates the progression of the cell cycle by being part of the G1/S phase checkpoint (Friend *et al.*, 1986). In *T. ni* Hi5 cells, transcription of *rb* increased after AcMNPV infection and reached a maximum at 8 h p.i. (Fig. 7.10), whereas expression of *rb* stayed constant during the time course of superinfection in C20 cells (Fig. 7.10). A similar trend for both cell lines was observed for the transcription of the *structural maintenance of chromosome 3* (*smc3*) after virus infection (Fig. 7.10). Moreover, *smc3* expression was reduced (0.52-fold) in unchallenged C20, when compared to uninfected *T. ni* Hi5 cells (supplementary CD Table S 7.3, 7.4). The Smc3 is part of the chromatid cohesion complex and holds sister chromatids together (reviewed by Strunnikov, 1998).

Additionally, key proteins for mitotic progression were decreased in C20 cells compared to *T. ni* Hi5 cells. This included genes encoding for the Aurora kinase (0.61-fold change),

surviving (0.40-fold change) and the cell division cycle protein 20 (CDC20: 0.41-fold change; Fig. 7.11; supplementary CD Table S 7.3, 7.4).

A decrease in expression of *rb* and other key mitotic genes may indicate that cell cycle progression and mitosis may be slowed compared to its parental cell line.

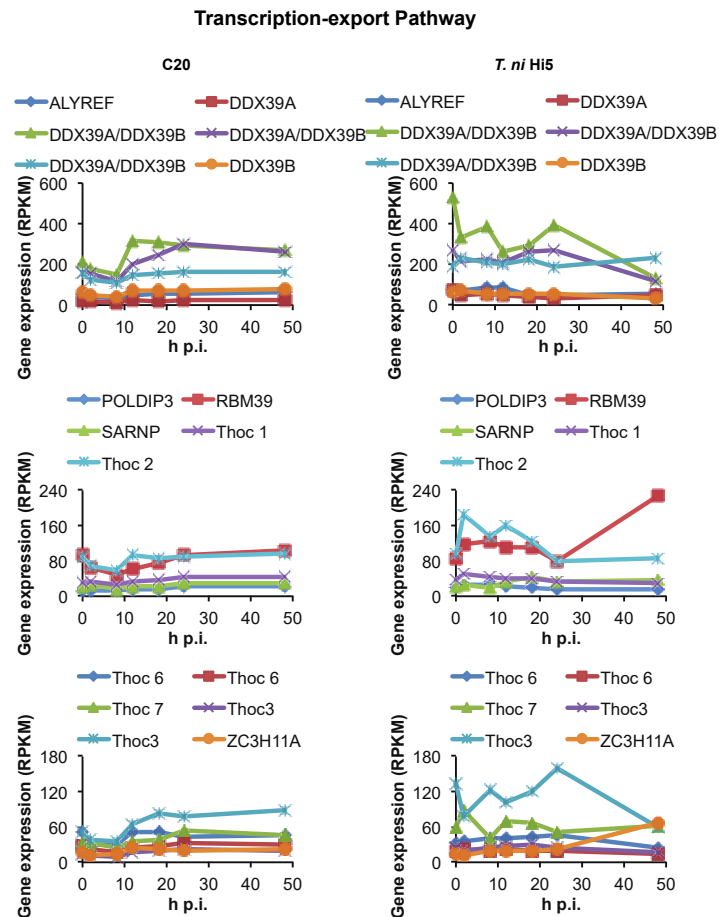


Figure 7.9 Transcription profiles of genes encoding for the Transcription export pathway

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: ALYREF: ally of AML-I and LEF-1, DDX: dead box protein, POLDIP3: polymerase delta-interacting protein 3, RBM: RNA-binding protein, SARNP: SAP domain containing ribonucleoprotein, Thoc: suppressor of transcriptional defects of hpr1 delta by overexpression complex, ZC3H11A: human zinc finger CCCH domain-containing protein 11A

7.5.9 Signalling

The transcription of some genes involved in MAPK signalling seemed to be decreased in unchallenged C20 cells, when compared to *T. ni* Hi5 cells. These genes include *Ras85D* (0.49-fold change), *MAPK4* (0.66-fold change) and *p39 MAPK* (0.56-fold change; supplementary CD Table S 7.3, 7.4). Additionally, expression levels of *Ras85D* remained similar over the time of superinfection in C20 cells, whereas its expression decreased over the time course of virus infection in *T. ni* Hi5 cell (Fig. 7.12). Also, the response of *p39*

MAPK to AcMNPV was delayed in superinfected C20 cells having a maximum at 24 h p.i. On the contrary, it reached a maximum at 12 h p.i. in virus-infected *T. ni* Hi5 cells (Fig. 7.12).

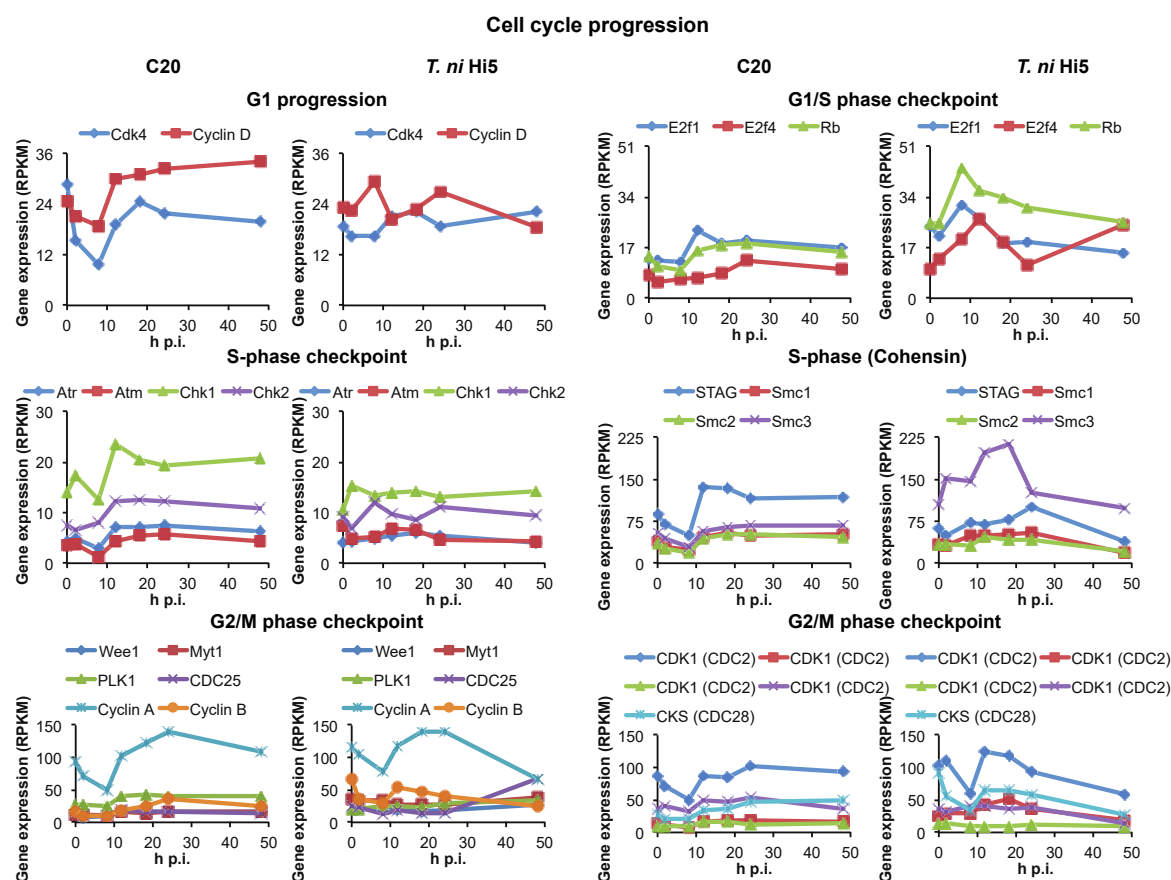


Figure 7.10 Transcription profiles of cell cycle progression-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Atm: ataxia telangiectasia protein, Atr: ataxia telangiectasia and Rad3-related protein, CDC: cell division cycle protein, Cdk: Cycline dependent kinase, Chk: checkpoint kinase, E2f: E2 transcription factor, G: gap, M: mitosis, PLK: polo-like kinase, Rb: retinoblastoma protein, S: synthesis, Smc: structural maintenance of chromosome

Also, JAK/STAT signalling in C20 cells differed from *T. ni* Hi5 cells (Fig. 7.13). While transcription of the *ubiquitin-conjugated enzyme 13 (UBC13)* was increased 2.2-fold, expression of the *gram-negative binding protein (GNBP)* and *SKPA* was reduced by 0.50-fold and 0.63-fold, respectively in unchallenged C20 cells compared to *T. ni* Hi5 cells (supplementary CD Table S 7.3, 7.4). Transcription of *DOMELESS* decreased after C20 superinfection until 8 h p.i. and subsequently reached the same level of expression as in unchallenged C20 cells, while transcription was decreased over the time of AcMNPV infection in *T. ni* Hi5 cells (Fig. 7.13). Expression of *HEMIPTEROUS* and *SKPA* reached a maximum at 12 h p.i. and 2 h p.i., respectively in virus-infected *T. ni* Hi5 cells, whereas the transcription levels did not change in C20 cells over the time of superinfection (Fig. 7.13).

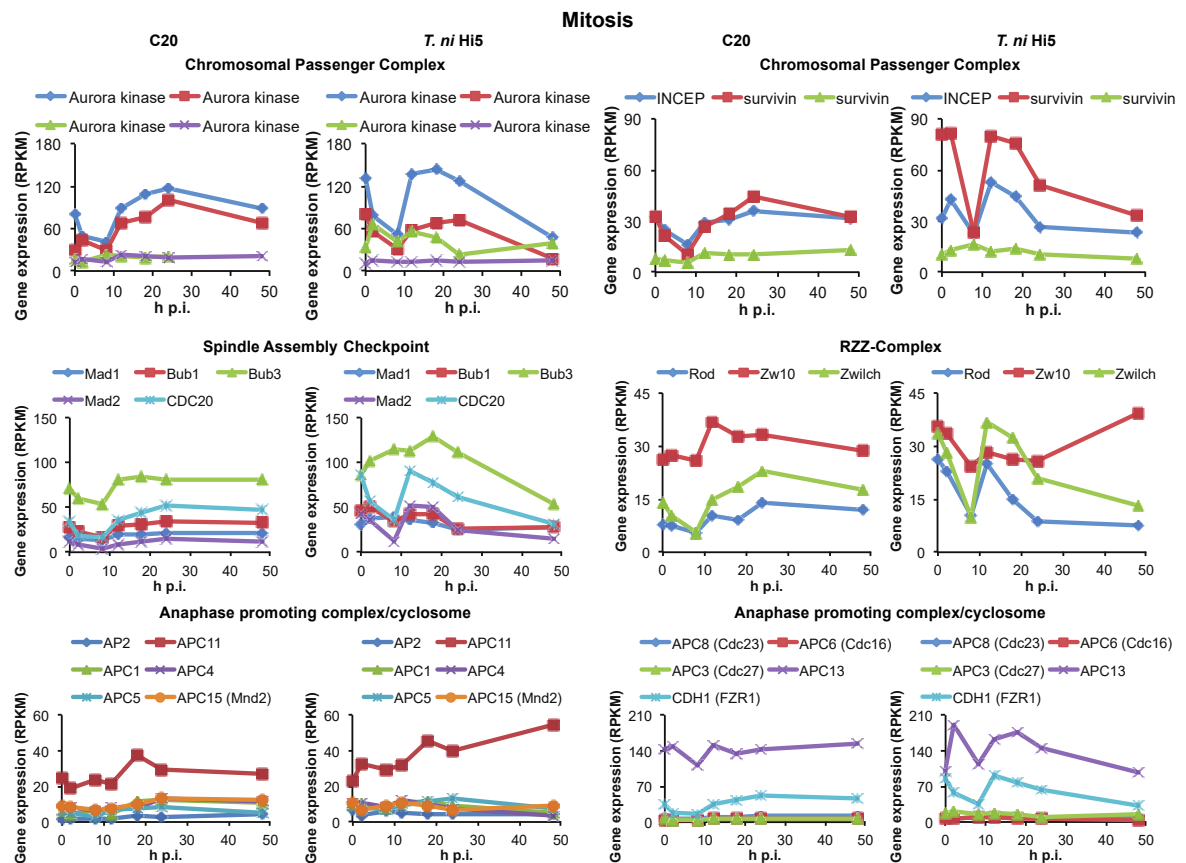


Figure 7.11 Transcription profiles of mitosis-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: AP: adapter protein, APCC: anaphase promoting complex/ cyclosome, Bub: budding uninhibited by benzimidazoles 1, BubR1. Bub related 1, CDC: cell division cycle protein, CDH: CDC20 homologue, CENP: centromere-associated protein, CPC: chromosomal passenger complex, INCEP: inner centromer protein, Mad: mitotic arrest deficient, Rod: rough deal, RZZ: Rod-ZW10, Zwlisch complex, SAC: spindle assembly checkpoint, Zw10: zeste white 10

Expression of *pi3k_68D*, the gene product of which is part of the PI3K signalling cascade, was reduced by 0.66-fold in unchallenged C20 cells compared to *T. ni* Hi5 cells (supplementary CD Table S 7.3, 7.4). Moreover, while *pi3k_68D* transcription increased from 2-18 h p.i. in virus-infected *T. ni* Hi5 cells, it decreased in AcMNPV-infected C20 cells until 8 h p.i. (Fig. 7.14).

Transcription of nuclear kappa-light-chain enhancer of activated B-cells (NF8kB19) was reduced 0.72-fold in C20 compared to *T. ni* Hi5 cells, and furthermore, expression slightly increased after the superinfection with AcMNPV in C20 cells. On the contrary, transcription of *NF8kB19* in *T. ni* Hi5 cells infected with AcMNPV decreased over the time course of infection (Fig. 7.15; supplementary CD Table S 7.3, 7.4).

Overall, cellular signalling in C20 cells was regulated differently than in *T. ni* Hi5 cells, which may lead to an alternative cellular response.

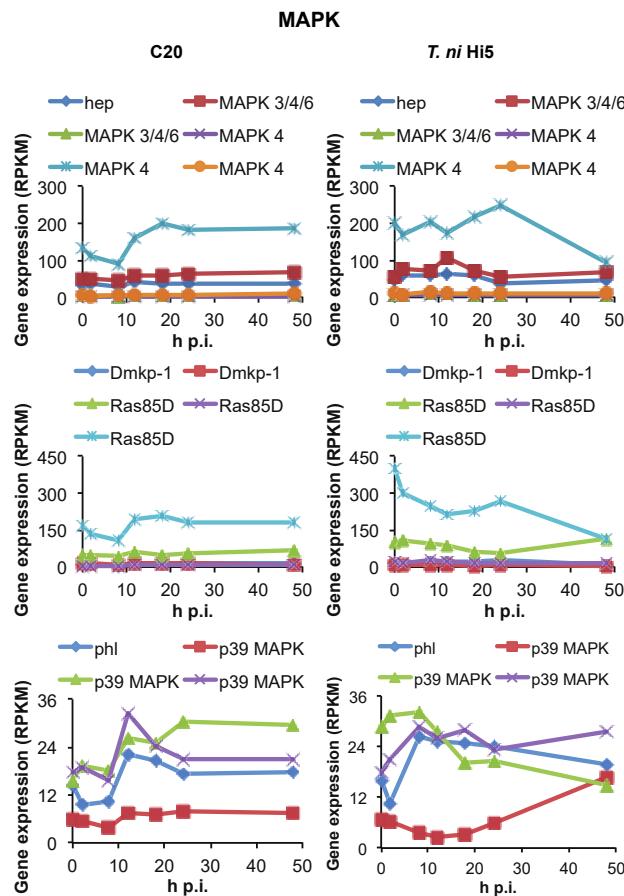


Figure 7.12 Transcription profiles of genes encoding for proteins involved in MAPK-signalling

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Dmcp: drosophila dual-specific phosphatase, MAPK: mitogen-activated protein kinase, Ras: Rat sarcoma

7.5.10 Stress response

Expression of a few members of the *hsp* family were reduced in C20 cells compared to *T. ni* Hi5 cells. These genes included *hsp21.4* (0.42-fold change), *hsp60* (0.59-fold change), *hsp70* (0.67-fold change) and *sHsp* (0.42-fold change; supplementary CD Table S 7.3, 7.4). On the other hand, transcription of *hsp20.8* was induced in C20 cells (supplementary CD Table S 7.4). Additionally, the transcriptional response of members of the *hsp* family differed after AcMNPV infection between *T. ni* Hi5 and C20 cells. While *hsp70* expression peaked at 18 h p.i. in virus-infected *T. ni* Hi5 cells, transcription did not change in AcMNPV-infected C20 cells (Fig. 7.16). Furthermore, transcription of *sHsp* declined in AcMNPV-infected *T. ni* Hi5 cells but stayed constant in superinfected C20 cells (Fig. 7.16).

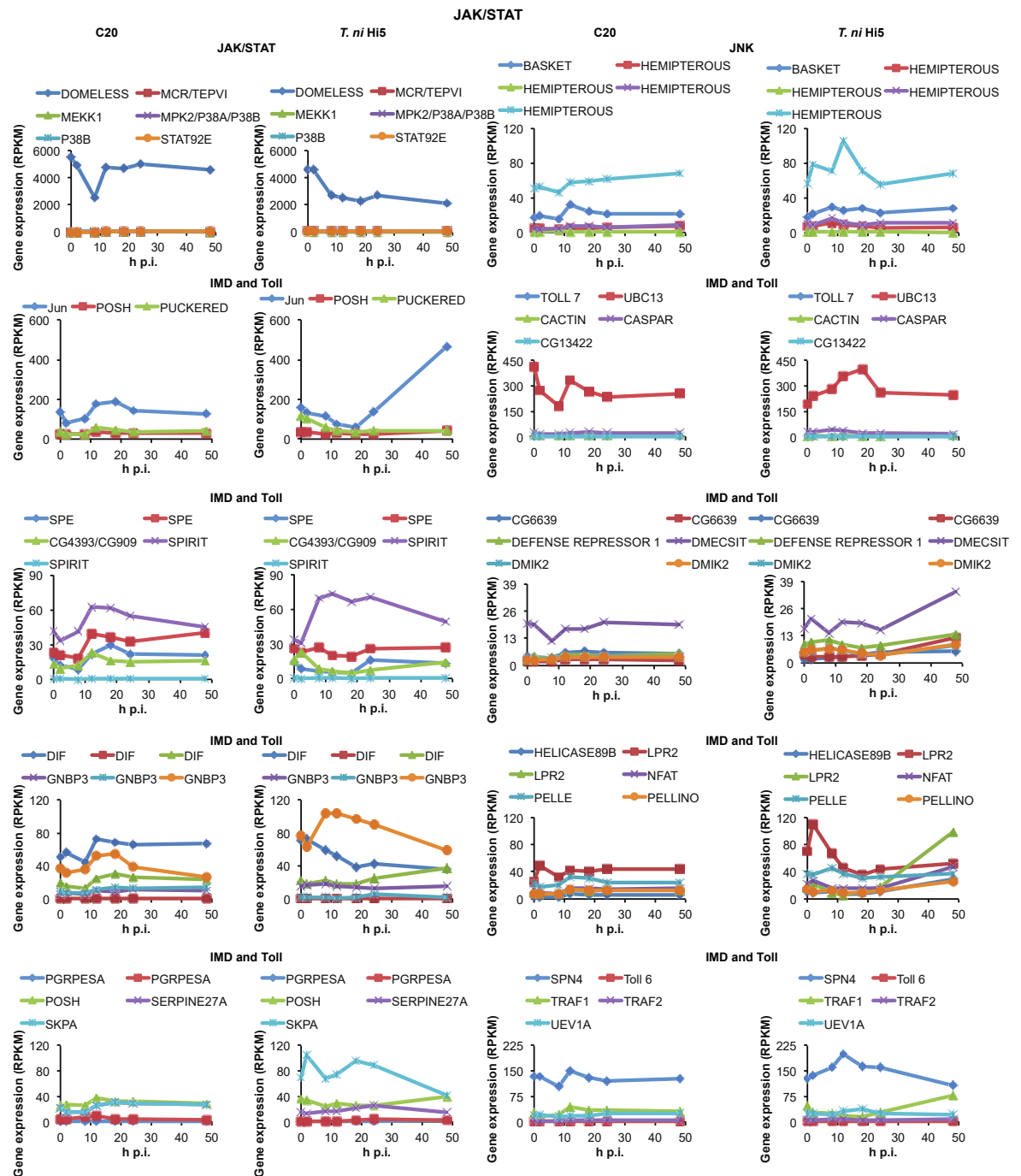


Figure 7.13 Transcription profiles of genes encoding for proteins involved JAK/STAT signalling

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: DIF: differential signalling, GNB3: gram-negative binding protein, JAK: Janus kinase, LPR2: LDL receptor homologous gene, MEKK: mitogen-activated protein kinase kinase 1, MPK: mitogen-activated protein kinase, NFAT: nuclear factor of activated T-cells, SPN: serpin, STAT: signal transducers and activators of transcription, TRAF: tumour necrosis factor receptor-associated factor, UBC: ubiquitin-conjugating enzyme

While the expression of the *catalase* was reduced 0.33-fold in C20 cells, transcription of the *superoxide dismutase* was 2-fold up regulated compared to *T. ni* Hi5 cells (supplementary CD Table S 7.3, 7.4). In addition, the transcriptional maximum of the *oxidative stress response gene* (OXSR) reached an earlier maximum at 12 h p.i. in

AcMNPV-infected C20 cells, whereas the maximum was not reached until 24 h p.i. in virus-infected *T. ni* Hi5 cells (Fig. 7.17).

In general, the stress response differed in C20 and *T. ni* Hi5 cells.

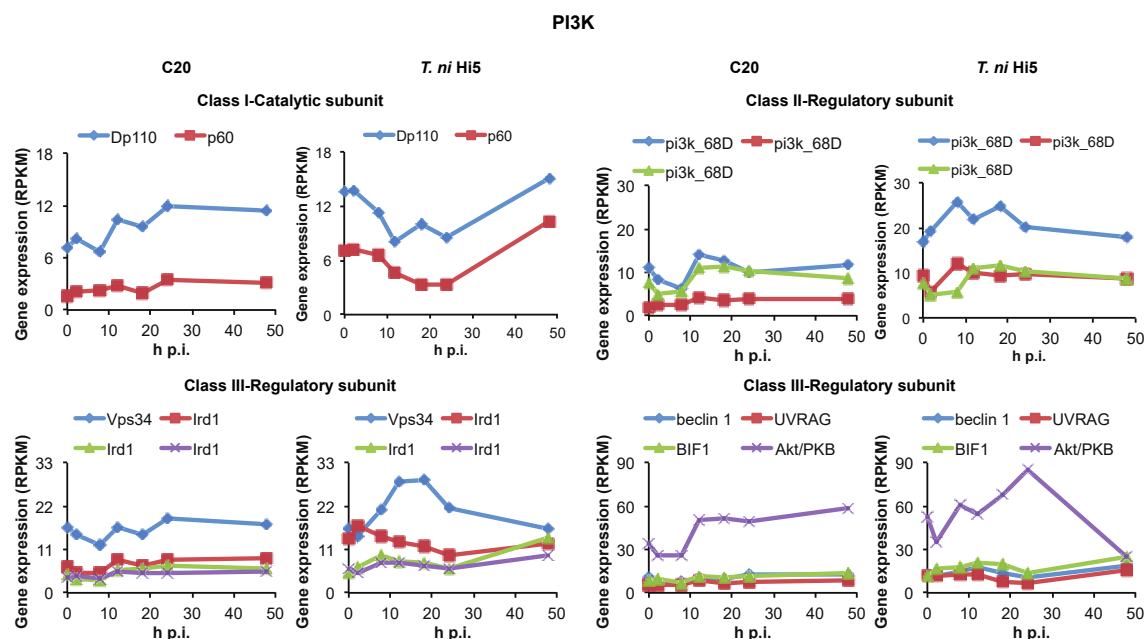


Figure 7.14 Transcription profiles of genes encoding for proteins involved in PI3K signalling

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Akt/PKB: protein kinase B, BIF: box-interacting factor, Ird: immune response-deficient, PI3K: phosphoinositide 3-kinase, UVRAG: UV irradiation resistance-associated gene, Vps: vacuolar protein-sorting associated protein

7.5.11 Antiviral response

Autophagy is a highly conserved lysosomal degradative pathway, which can also serve as an antiviral defence mechanism (reviewed by He and Klionsky, 2009; Wileman, 2013; Viret *et al.*, 2018). Although studies on autophagy in AcMNPV-challenged cells are rare (Wei *et al.*, 2012), it is an interesting hypothesis if the persistent virus in C20 can be maintained because the antiviral defence mechanisms were down regulated or if C20 cells are resistant to superinfection as a consequence of an up regulation of autophagy-related genes.

Genes involved in vesicle induction (*Atg101*, *Atg17*), vesicle elongation (*Atg4B*, *Atg12*) and signalling (*Vps20*, *Ras*) were repressed in C20 cells (supplementary CD Table S 7.4). Moreover, expression of *Atg8*, *Atg3* and *Tsc* was reduced (by ~ 0.5-fold) in C20 cells compared to *T. ni* Hi5 cells indicating that autophagy may not play a role in the exclusion of superinfection but might be important for the maintenance of baculovirus persistence (Fig. 7.18; supplementary CD Table S 7.3, 7.4).

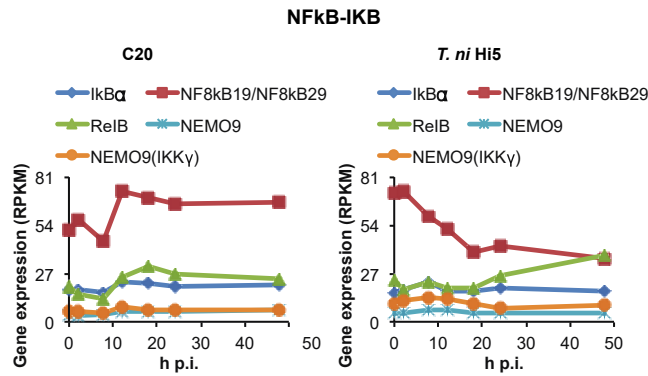


Figure 7.15 Transcription profiles of genes encoding for proteins involved in NFkB-IkB signalling

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: IKK: IκB kinase, NEMO: nuclear factor-κB essential modulator, NFκB: nuclear factor kappa-light chain enhancer of activated B-cells, Rel: Rel-like domain containing proteins

The RNA interference pathway plays a major role as an antiviral defence mechanism by the posttranslational silencing of transcribed genes (Ratcliff *et al.*, 1999; Keene *et al.*, 2004; Andino Ronald van Rij *et al.*, 2006). The expression of two of the key genes involved in RNAi were down regulated in C20 cells compared to *T. ni* Hi5 cells. These genes include *Arg2* (0.56-fold change), which interacts with siRNAs to form the RNA-induced silencing complex (RISC) and *Dicer-2* (0.45-fold change) that cleaves dsRNA into short RNAs (supplementary CD Table S 7.3, S 7.4; reviewed by Sashital and Doudna, 2010). Interestingly, expression of RNAi-related genes were overall down regulated during the time course of virus infection in *T. ni* Hi5 cells, while transcription of these genes remained constant in AcMNPV-infected C20 cells (Fig. 7.19). This may suggest that the RNAi pathway as an antiviral defence mechanism was affected (down regulated) in C20 cells in a way that enabled the baculovirus to persist.

The third most common antiviral defence mechanism is the induction of apoptosis after virus infection (Clem *et al.*, 1991; Crook *et al.*, 1993; reviewed by Tschopp *et al.*, 1998). A gene that was up regulated during apoptosis and the product of which translocates from the cytoplasm to the nucleus to activate other apoptotic related transcription factors was the *death-associated inhibitor of apoptosis* (PDCD5; Li *et al.*, 2016). In C20 cells transcription of this gene was 0.68-fold down regulated, when compared to *T. ni* Hi5 cells (supplementary CD Table S 7.3, 7.4). While AcMNPV-infection strongly activated its transcription in *T. ni* Hi5 cells, expression of PDCD5 only slightly increases after virus-infection in C20 cells (Fig. 7.20). In addition, expression of *caspase-1*, which activates the programmed lytic cell death (Miao *et al.*, 2011), was reduced 0.33-fold in C20 cells (supplementary CD Table S 7.3, 7.4). Apoptotic stimuli usually cause the release from Cytochrome C from the mitochondria resulting in caspase activation and cell death

(reviewed by Jiang and Wang, 2004). While AcMNPV infection in *T. ni* Hi5 cells stimulated *Cytochrome C* transcription and reached a maximum at 12 h p.i., its expression did not change in virus-infected C20 cells (Fig. 7.20). Overall, this indicated that the apoptotic response in C20 cells was reduced compared to *T. ni* Hi5 cells.

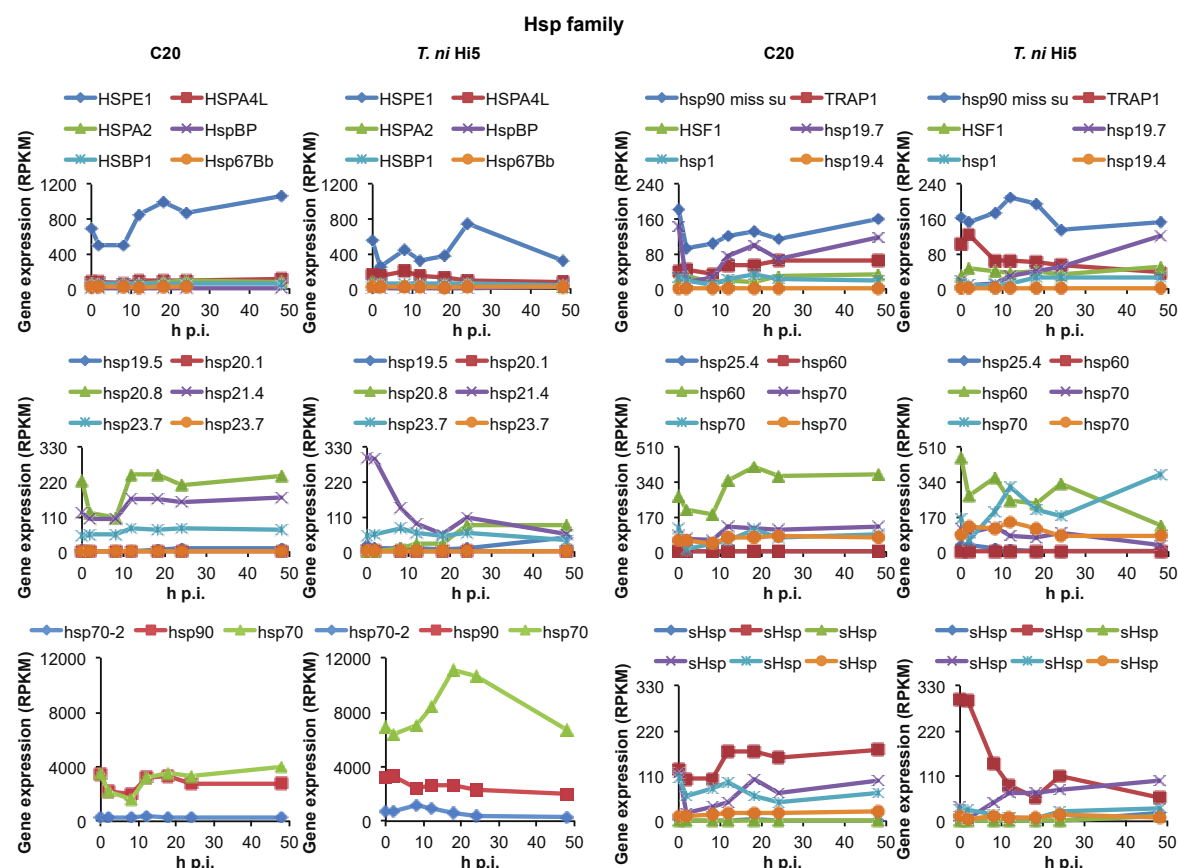


Figure 7.16 Transcription profiles of Hsp family-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Hsp: heat shock protein, sHSP: small heat shock protein

7.6 Discussion

To understand the specific features of virus-host interaction that permit persistent baculovirus infections and how both the virus and the host evolved to maintain the fine balance of host cell transcription and virus replication, Illumina® deep sequencing of the virus (Chapter 6) and host transcriptome was performed.

While the total percentage of reads mapped to the *T. ni* genome continuously decreased over time down to ~6% after AcMNPV infection in *T. ni* Hi5 cells, host mRNA from C20 cells made up >95% of total mRNA. Even when C20 cells were superinfected with AcMNPV >90% of the total mRNA derived from host gene expression (Fig. 7.2), indicating that host transcription was not shut down in C20 cells (Table 7.1). However, ~8-17% of host genes were differentially expressed in C20 cells when compared to its parental cell

line (Table 7.2), suggesting that host gene expression was regulated in a different way in a persistent baculovirus infection than in uninfected cells.

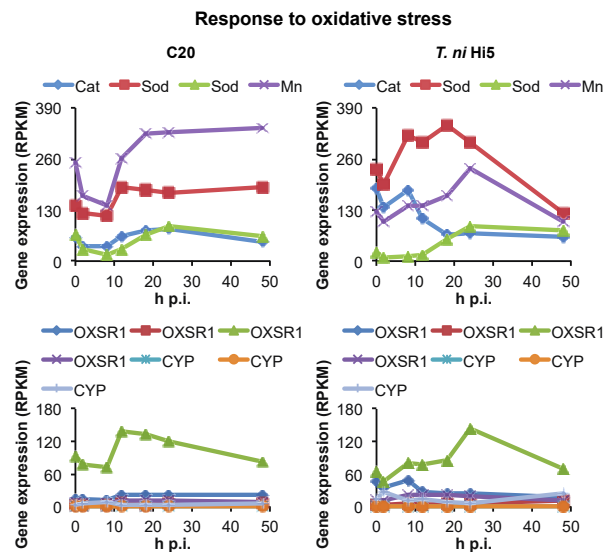


Figure 7.17 Transcription profiles of response to oxidative stress-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Cat: catalase, CYP: cytochrome P450, Mn: superoxide dismutase 2, OXSR: oxidative stress response, Sod: superoxide dismutase 1

Surprisingly, host gene expression was not significantly down regulated in AcMNPV-infected *T. ni* Hi5 cells and only ~5% of host transcripts were considered as significantly reduced at 48 h p.i. (Table 7.1). A microarray-based method to investigate the impact of AcMNPV on its host determined that 41% of *Sf21* genes were down regulated at 24 h p.i. compared to uninfected cells (Salem *et al.*, 2011). Similar results were observed after AcMNPV infection in both *Sf9* and *Sf21* cells and analysis using Northern blot analysis (Ooi and Miller, 1988; Nobiron, 2003) as well as RNA-seq analysis of the virus-infected *T. ni* cell line Tnms42 (Chen *et al.*, 2014). However, no significant changes in host gene expression as a response of AcMNPV infection in *T. ni* Hi5 cells might be observed in this study as the standard deviation of the samples collected at 12-18 h p.i. were high (supplementary CD Table S 7.2). At later time points, virus-infected cells are more prone to lyse and so there RNA content lost to the medium. Extraction of RNA from this medium would have been interesting.

To gain a more detailed picture of host gene expression in C20 cells, differentially expressed host genes were identified by comparing C20 host gene expression with transcription of *T. ni* Hi5 genes in mock-infected cells (Table 7.2-7.5). As only ~1/3 of the *T. ni* genome could be annotated, most of the DGE host genes in C20 cells could not be ascribed a function.

Interestingly genes encoding for proteins (larval pupal rigid cuticle protein, nidogen and shootin-1) required for the *in vivo* development of the insect were down regulated (Table

7.5). While nidogen is needed for the basal lamina assembly (reviewed by Yurchenco and O'Rear, 1994), shootin-1 is required for neuronal polarisation (Toriyama *et al.*, 2006). It may be speculated that C20 cells evolved in a way to not waste resources on nonessential gene expression.

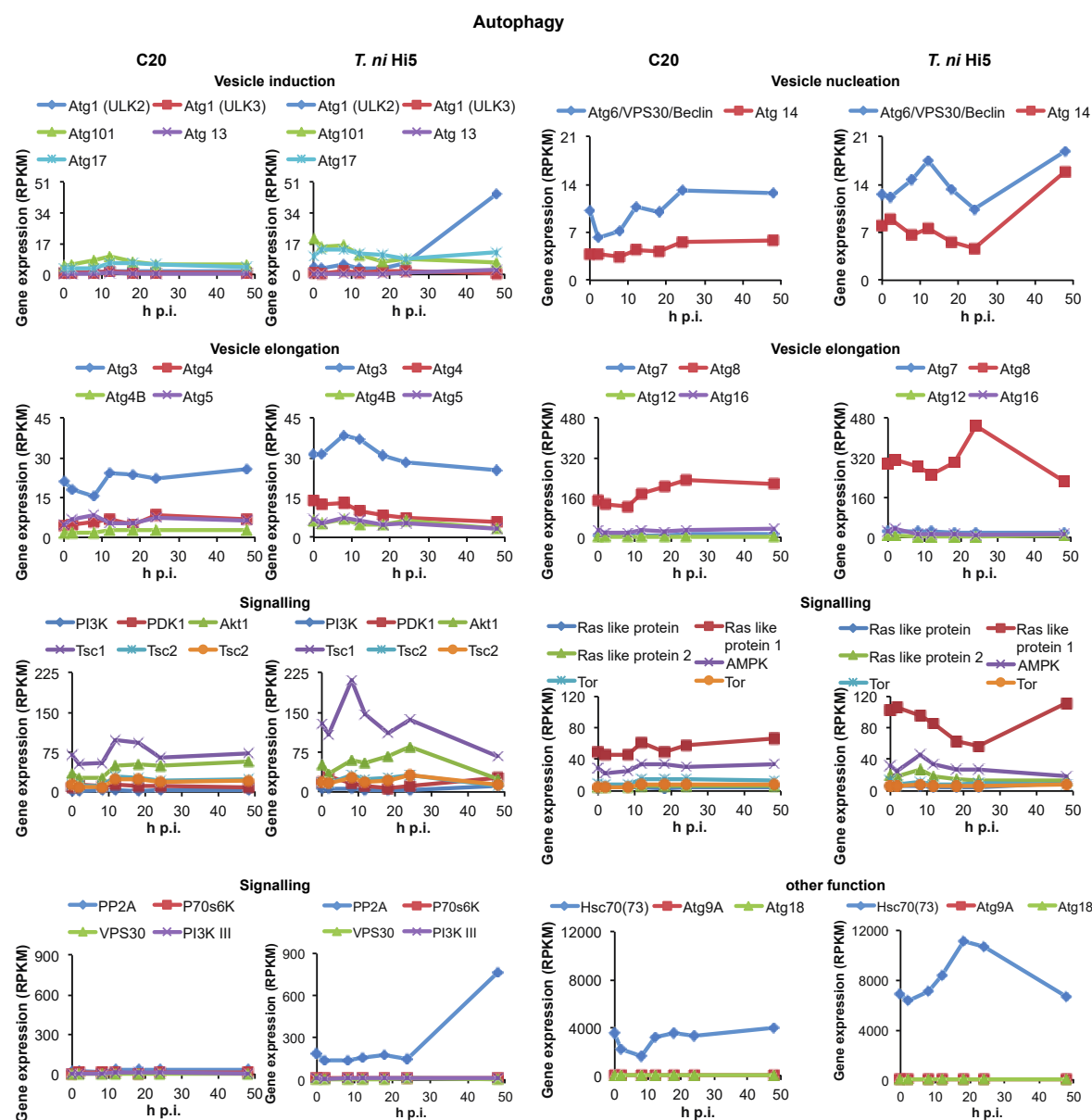


Figure 7.18 Transcription profiles of autophagy-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Atg: autophagy related gene, Akt: protein kinase B, AMPK: AMP-activated protein kinase, P70s6K: ribosomal protein S6 kinase, HSC: heat shock protein, PDK: protein kinase D, PI3K: phosphoinositide-3-kinase, PP2A: protein phosphatase 2, TSC: tuberous sclerosis complex, Tor: target of rapamycin, ULK: Unc-5 like autophagy activating kinase, VPS: vacuolar protein sorting-associated protein

The most highly up regulated host genes in C20 cells were GSTs (Table 7.3). Interestingly, in AcMNPV-infected Tnms42 cells GST was found to be one of the most highly repressed genes (Chen *et al.*, 2014) pointing out a difference in gene transcription

between a productive and persistent baculovirus infection. In general, GST is an enzyme that catalyses the conjugation to a variety of electrophilic substances and thereby contributing to the detoxification of the cell. Additionally, GST is involved in the regulation of the apoptotic pathway. It has a potential role in apoptosis resistance by suppressing activation of the JNK signalling pathway, which can be activated by stress targeting transcription factors involved in cell proliferation and cell death (Romero *et al.*, 2006; reviewed by Laborde, 2010). On the contrary to the persistent baculovirus in C20 cells, the enterovirus Coxsackievirus B3 phosphorylates JNK to increase viral progeny release (Si *et al.*, 2005).

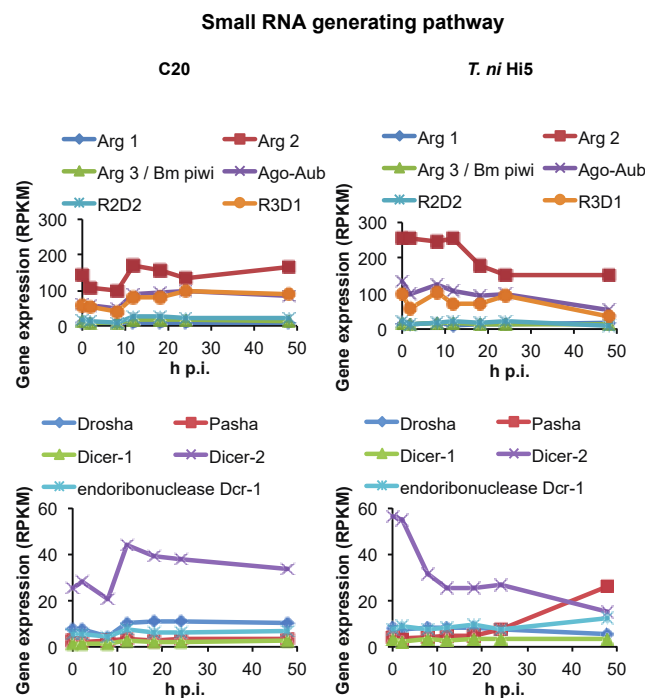


Figure 7.19 Transcription profiles of genes encoding for RNAi-related proteins

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviations: Arg: protein argonaute

In addition to the potential inhibition of apoptosis in C20 cells by GST and JNK signalling, expression of the apoptotic genes inducing *caspase-1* and *PDCD5* were reduced (Fig. 7.20). In contrast to lytic virus infections that only need to prevent apoptosis until the completion of the viral replication cycle, persistent viruses have to inhibit the apoptotic response permanently. The Epstein-Bar virus (EBV) belonging to the human herpesviruses and persistently infecting human B lymphocytes encodes for eight latent proteins. When all eight EBV latent proteins are expressed, B-cells were resistant to apoptosis and enhanced cell survival was mediated (Gregory *et al.*, 1991). The alphabaculoviruses genome encodes for three anti-apoptotic proteins: P35, IAP-1 and IAP-2 (Clem *et al.*, 1991; Crook *et al.*, 1993). Interestingly, in *Sf* cells caspase-1 was able to induce apoptosis and moreover to cleave P35. Furthermore, caspase-1 activity might

be inhibited by P35 (Ahmad *et al.*, 1997). However, *T. ni* Hi5 cells do not develop apoptosis in response to *p35* mutants (Hershberger *et al.*, 1992; Gross and Shuman, 1998; Griffiths *et al.*, 1999).

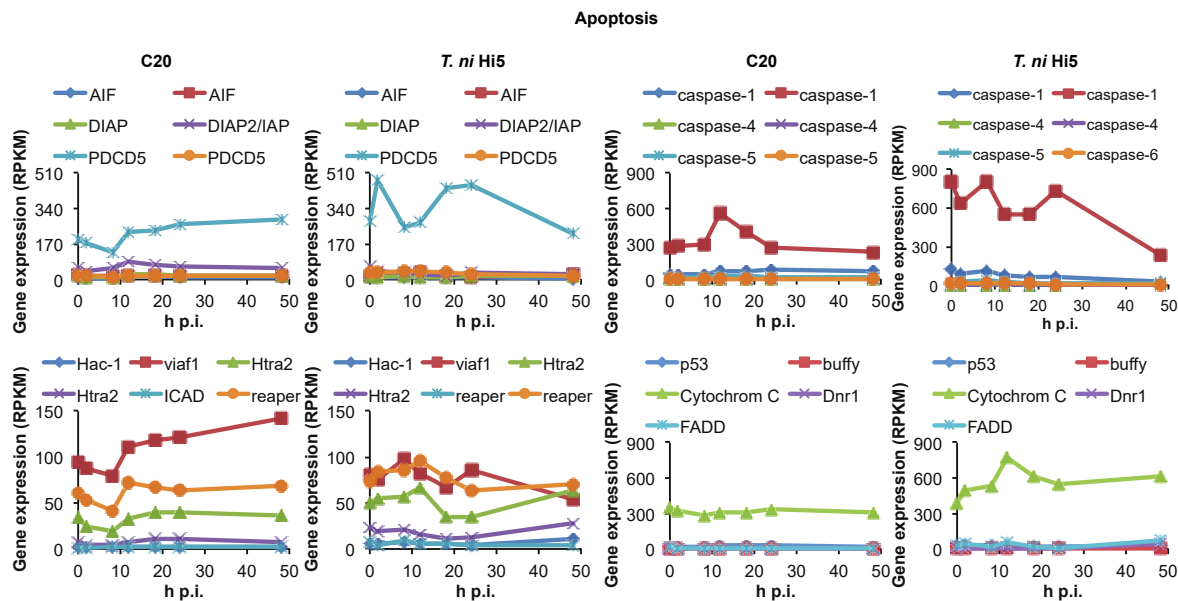


Figure 7.20 Transcription profiles of apoptosis-related genes

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.; Abbreviation: AIF: apoptosis inducing factor, DIAP: death-associated inhibitor of apoptosis, Dnr: defence repressor, FADD: Fas-associated protein with death domain, IAP: inhibitor of apoptosis, ICAD: caspase-activated DNase, viaf: IAP-associated factor

The second antiviral defence mechanism that was analysed in more detail was the RNAi pathway. A productive virus infection reduced expression of genes involved in the RNAi pathway over the time course of infection (Fig. 7.19; Jayachandran *et al.*, 2012; Chen *et al.*, 2014). In C20 cell RNAi-related genes, especially *arg-2* and *dicer-2*, were down regulated when compared to its uninfected parental cell line (Fig. 7.19; supplementary CD Table S 7.3, 7.4). Both gene products are the key components of the insect siRNA-mediated posttranslational silencing process (reviewed by Bronkhorst and van Rij, 2014). In general, virus infections can act as drivers of evolution (Swevers *et al.*, 2013). Among the most rapid evolving genes in *D. melanogaster* and the mosquito *Aedes aegypti* are *arg-2* and *dicer-2*, which undergo a rapid, positive selection. They evolve significantly faster than other immune system genes or the genome as a whole and thereby significantly contributing to host-pathogen interactions (Fig. 7.19; Obbard *et al.*, 2006; Bernhardt *et al.*, 2012).

Autophagy is a cellular response to defend the cells against invading pathogens. For example, the induction of autophagy suppressed the survival of group A *Streptococcus* (also known as *S. pyogenes*) and *Mycobacterium tuberculosis* (Gutierrez *et al.*, 2004;

Nakagawa *et al.*, 2004). However, it appears that AcMNPV did not evolve a mechanism to inhibit autophagy during a productive infection (Fig. 7.18; Wei *et al.*, 2012). On the contrary, C20 cells reduced the expression of autophagy-related genes and thereby potentially increasing the survival of intracellular virions (Fig. 7.18).

Overall, genes involved in the host antiviral response were down regulated, potentially accounting for the maintenance of the persistent baculovirus in C20 cells (Fig. 7.18-7.20).

Generally, baculovirus infections cause protein and membrane lipid oxidation and therefore increase the level of oxidative stress, which can contribute to cell death (Wang *et al.*, 2001). However, the expression of manganese superoxide dismutase (MnSOD) significantly reduced oxidative damage in virus-infected *T. ni* Hi5 but not *Sf9* cells (Wang *et al.*, 2004). Expression of *SOD* genes in C20 cells were expressed but were regulated differently than in *T. ni* Hi5 (Fig. 7.17).

A second group of genes that responds to cellular stress are *Hsps* (reviewed by Feder and Hofmann, 1999). Infection with AcMNPV up regulates expression of *Hsp70* in *Tnms42*, *Sf21* and *Hv-Am1* cells, which derived from *Heliothis virescens* (Popham *et al.*, 2010; Salem *et al.*, 2011; Chen *et al.*, 2014). Also, in C20 cells *Hsp70* was one of the most up regulated host genes compared to uninfected *T. ni* Hi5 cells (Table 7.3). Even superinfection of C20 cells manipulated the expression of *Hsps* (Fig. 7.16). Hence, its increased expression was not a unique feature of the C20 cell line but for baculovirus infections. In general, the heat shock response is important for baculovirus replication (Fig. 7.16; Lyupina *et al.*, 2010). Inhibition of HSP90 by a pharmaceutical drug decreased the rate of viral DNA synthesis further supporting the importance of HSPs during the viral replication cycle (Lyupina *et al.*, 2011).

Both ROS and *Hsps* are part of the signal transduction network in cells (reviewed by Feder and Hofmann, 1999; D'Autréaux and Toledano, 2007). The regulation of signalling is essential to maintain a balanced transcriptional control (reviewed by Weitzman and Yaniv, 1998). If the signalling cascades are altered as in C20 cells (Fig. 7.12-7.15) this will change the maintenance of the transcriptional control and will ultimately lead to a different cell response (Fig. 7.3-7.13).

Altogether, a different host transcriptome response of all analysed cellular pathways required for the viral replication cycle (including Lipid synthesis, ESCRT, SNARE, NPC, HDAC, TREX, cell cycle progression and mitosis) could be observed in C20 cells compared to *T. ni* Hi5 cells (Fig. 7.3-7.13, Table 7.2-7.5). Particularly interesting was the down regulation of *HDAC1* in C20 cells (Fig. 7.8) indicating that transcriptional regulation of C20 cell may be executed on chromatin level organisation. A persistent virus that limits

its gene expression during its latent state by the regulation of chromatin structures is HSV. On one site, the HSV lytic-gene promoters form a complex with modified histones that are associated with heterochromatin causing silencing of the specific lytic genes. On the other site, the latency permitting promoter LAT is increasingly associated with acetylated H3 histone and therefore with the transcriptional active euchromatin (Kubat *et al.*, 2004; Wang *et al.*, 2005). It would be interesting to investigate the regulation of transcriptional control in C20 cells in more detail, e.g. by Chromatin ImmunoPrecipitation DNA-sequencing (ChIP-seq).

Furthermore, genes encoding for mitotic checkpoint proteins and cell cycle progression were down regulated in C20 cells compared to its parental cell line (Fig. 7.10, 7.11). Virus-infected *Sf9* cells are arrested in G2/M phase of the cell cycle potentially by an AcMNPV encoding protein with CDC-kinase activity (Braunagel *et al.*, 1998). When *Sf21* or TN-368 cells were transfected with *ie-2*, the cell cycle arrest occurred in the S-phase and the arrest was dependent on the integrity of the IE-2 RING finger motif (Prihod'ko and Miller, 1998). Additionally, it was determined that AcMNPV targets at least two cell cycle checkpoints depending upon the phase in which virus infection occurs. Virus-infected *Sf9* cells in G2/M phase were unable to undergo mitosis, while cells infected either in G1 or S-phase were arrested in S-phase (Ikeda and Kobayashi, 1999). The virus-mediated regulation of the cell cycle by targeting checkpoints proteins is an often observed phenomena, e.g. from Adenoviruses, human cytomegalovirus or hepatitis B virus (Kim *et al.*, 2008; Mui *et al.*, 2010; Fehr *et al.*, 2012). It may be hypothesised that the down regulation of cell cycle proteins may account for the longevity in C20 cells.

An interesting phenotype that was observed in the context of the C20 cell line was that only a minority of cells could be infected with a second AcMNPV. The exclusion of superinfection in C20 cells occurs early potentially during the binding step of the virus to its host cell (R. Arinto Garcia and R. D. Possee, personal communication). Two cellular pathways may be particularly interesting in this context. These pathways include the CME and Lipid synthesis pathway. Genes encoding for proteins required for the endocytic pit formation were repressed and expression of some of the key proteins were down regulated (Fig. 7.3; Table 7.4). Interestingly, virus interference in Vesicular Stomatitis Virus (VSV) was associated with both a decreased rate of formation of endocytic vesicles and a reduced rate of virus internalisation (Simon *et al.*, 1990). It would be interesting to verify if the CME pathway is down regulated in C20 cells by the study of the uptake of other ligands (e.g. transferrin). In addition, gene transcription encoding for the fatty acid synthesis and glyceride synthesis were altered in C20 cells compared to its parental cell line (Fig. 7.3). This may result in a variation of the plasma membrane composition and fluidity and may therefore affect virus entry (Chernomordik *et al.*, 1995).

That the membrane composition may be altered in C20 cells was also indicated by various differentially expressed genes (Table 7.2-7.4). Transcription of both the *acyl-desaturase* and *trehalose transporter* were the most up regulated in C20 cells compared to *T. ni* Hi5 cells (Table 7.3). The acyl-desaturase is part of the fatty acid biosynthesis and plays a key role in the maintenance of the structure of cellular membranes by the regulation of the level of unsaturated membrane lipids (reviewed by Los and Murata, 1998). Trehalose is a non-reducing disaccharide of glucose and stabilises lipid bilayers (reviewed by Elbein *et al.*, 2003; Tang *et al.*, 2007). Furthermore, expression of genes encoding for various transmembrane channels were induced (glutamate-gated chloride channel and acetylcholine receptor; Table 7.2). Transcription of *caveolin-3*, the gene product of which is required to form membrane invaginations, the so-called caveolae, was repressed (Table 7.4). Caveolin does not only play a role in endocytosis and cellular signalling, but is also required for maintaining the membrane lipid composition (reviewed by Cheng and Nichols, 2016). Virus-mediated modulation of the lipid metabolism required for their replication has been also observed for other viruses, e.g. vaccinia virus (VV), hepatitis C virus (HCV) or human cytomegalovirus (HCMV; reviewed by Mazzon and Mercer, 2014).

Furthermore, expression of an *ezerin radixin moesin* (erm) homolog was one of the most significant down regulated host genes in C20 cells (Table 7.5). The ERM proteins usually regulate membrane-associated signalling pathways, thereby modulating the cortical actin cytoskeleton response (reviewed by Neisch and Fehon, 2011). A modulation of the actin cytoskeleton in C20 cells may be of particular interest as virus interference in alphabaculovirus infection is associated with a reorganisation of the actin cytoskeleton. Inhibition of actin polymerisation by Chytochalasin D resulted in a successful superinfection of SfMNPV in AcMNPV-infected Sf9 cells (Beperet *et al.*, 2014).

It may be further speculated that the superinfection occlusion may act on several levels and may involve several cellular pathways in C20 cells. To ensure the exclusion of superinfection in bovine viral diarrhoea virus (BVDV) virus interference acts on at least two levels, virus entry and virus replication (Lee *et al.*, 2005).

Overall, the involvement of the various cellular pathways that were analysed in the context of the maintenance of a persistent baculovirus infection needs to be determined and confirmed experimentally. Moreover, how translational processes or posttranslation modification may account for the maintenance of a persistent baculovirus in C20 cells is still unknown. In addition, the analysis performed on the viral and the host transcriptome in C20 cells was an average of the whole cell population. As phenotypic differences were observed in a single C20 population (already discussed in Chapter 6), it would be

interesting to investigate if all C20 cell contain and express the persistent baculovirus and how gene expression is regulated in a single cell, e.g. by single cell RNA-seq.

7.7 Conclusion

The aim of this chapter was to determine how the host transcriptome of C20 cells that contains a persistent baculovirus infection was regulated when compared to a lytic baculovirus infection. Genes encoding for various cellular pathways required for virus replication, the host stress response and the antiviral defence mechanisms were regulated differently in C20 than in its parental cell line. This potentially leads to the maintenance of the persistent baculovirus and does not induce cell lysis as in an overt infection. Overall, it can be hypothesised that the C20 cells evolved together with the persistent baculovirus leading to a modulation of cellular pathways and a specific balance of virus and host gene expression.

Chapter Eight:

**The establishment of an insect
cell line harbouring a
persistent baculovirus**

8.1 Introduction

The C20 cell line was established over seven years ago when *T. ni* Hi5 cells became persistently infected with AcUW1.*lacZ*. Further studies showed that insect cell lines harbouring other persistent baculoviruses could also be generated expressing a red fluorescent protein (dsRed), urokinase, influenza virus neuraminidase and even wild type AcMNPV. Unfortunately, these persistently-infected cell lines were not stored over the years (R. D. Possee, personal communication). Moreover, although the establishment of persistently infected insect cell lines worked very well at the beginning, after a break was undertaken in the studies, it was not possible to regenerate these cell lines. It was even impossible to recreate the C20 cell line although the same virus seeding stock and the same cells (same passage of cells) were utilised (R. Arinto Garcia, R. D. Possee, personal communication).

It was hypothesised that recent batches of the cell culture medium were formulated in a way that increased BV production after virus infection, albeit serendipitously. Hence, the insect cells would be overloaded with BVs. Moreover, an enhanced BV production would ultimately induce cell lysis in every single cell after baculovirus infection. Therefore, the crucial balance of host and virus gene expression that is unique for an insect cell line harbouring a persistent baculovirus could not be established. It was considered if a reduced BV production of the virus might permit a persistent baculovirus infection in insect cells. To test this hypothesis a virus, AcLEF-2^{C84A}, was utilised that produced 10-fold fewer BVs compared to wt-infected cells (Chapter 4; Fig. 4.19D).

8.2 Establishment of *T. ni* Hi5 cells harbouring a persistent AcLEF-2^{C84A} infection

To establish a persistent baculovirus infection, 0.5×10^6 *T. ni* Hi5 cells were seeded into 35 mm dishes and subsequently infected with AcLEF-2^{C84A}. It was decided to infect the cells with various low MOIs (between MOI 0.01-1.5). The reason for the low MOI was to allow multiple cycles of virus infections and moreover, to provide more time for the insect cells to adapt to a persistent baculovirus infection.

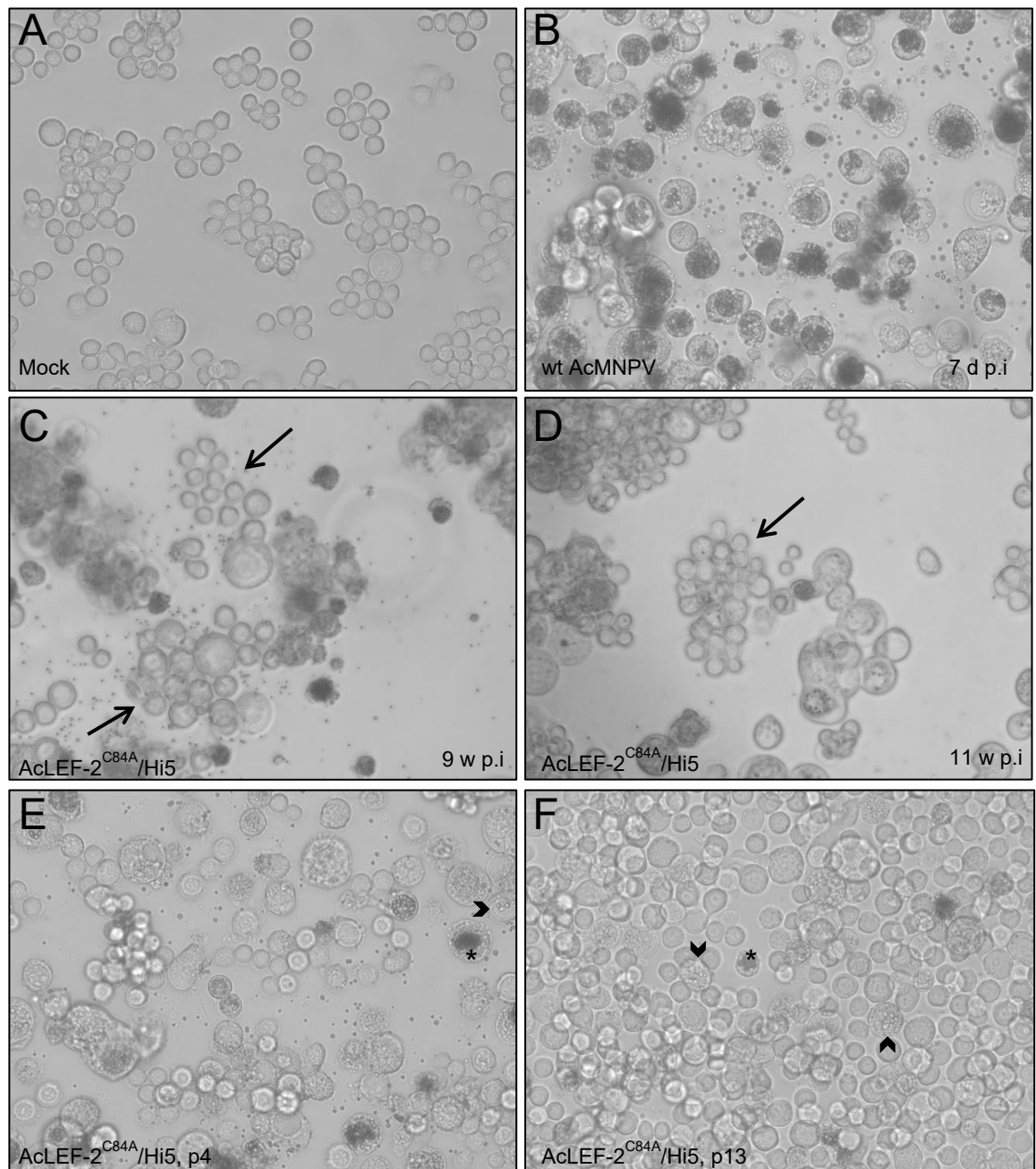


Figure 8.1 Establishment of a persistent AcLEF-2^{C84A}/Hi5 cell line

T. ni Hi5 cells were A) mock-infected or B) infected with wild-type (wt) AcMNPV (MOI 3) generating a lytic virus infection and polyhedra release at 7 d. p.i. C-D) Cells survived the initial infection with AcLEF-2^{C84A} (MOI 0.05) establishing a persistent virus infection in *T. ni* Hi5 cells a few weeks (w.) p.i. Arrows highlight healthy looking newly grown clusters of cells. E-F) Early passage of the newly established AcLEF-2/Hi5 cell line. Arrowheads highlight cells with a few polyhedra (FP) phenotype, while stars point at cells containing a wt polyhedra phenotype.

Usually, AcMNPV infection causes cell lysis and polyhedra release at the end of the virus replication cycle *in vitro* (Fig. 8.1B). However, one culture (infected with MOI 0.05) survived the initial virus infection and clusters of healthy looking, shiny cells grew after a few weeks p.i. (Fig. 8.1C, D; compare to mock-infected *T. ni* Hi5 cells Fig. 8.1A). The surviving *T. ni* Hi5 culture containing the persistent AcLEF-2^{C84A} baculovirus was passaged and amplified in monolayer culture. Occasionally, the persistent virus became

activated in some culture flasks resulting into a lytic baculovirus infection (data not shown).

Early AcLEF-2^{C84A}/Hi5 passages (e.g. p4 Fig. 8.1D) contained healthy looking cells but in addition, some cells accumulated polyhedra inside their nuclei. Those were released upon cell lysis. Hence, it is very likely that AcLEF-2^{C84A}/Hi5 contained a mixed population of viruses. At cell culture passage 13, fewer polyhedra were observed in the cell culture medium of the newly established persistently infected cell line. Moreover, most nuclei containing polyhedra showed a FP phenotype in p13 (Fig. 8.1E arrowheads). However, even some cells in p4 contained a FP phenotype (Fig. 8.1D, arrowhead).

8.3 FP-phenotype of AcLEF-2^{C84A}/Hi5 cells

To analyse the FP phenotype in more detail the virus DNA from the clarified cell culture medium of AcLEF-2^{C84A}/Hi5 p13 was extracted, the *fp25k* orf was PCR amplified and analysed by Sanger sequencing (Source Bioscience, data not shown). Sequencing confirmed an A insertion within a run of seven existing A's resulting in a premature stop codon. Therefore, *fp25k* in p13 was expressed as a truncated protein (63 amino acids). However, there might be more mutations inside *fp25k*, which could not be determined accurately (data not shown). The sequence assembly inside the trace files generated multiple chromatogram peaks for single bases. Therefore, the nucleotide sequence could not be determined (data not shown). As a control the virus stock of AcLEF-2^{C84A} that was used for the generation of the persistent cell line and in addition AcLEF-2^{C84A}/Hi5 p1 were analysed for a *fp25k* mutation by Sanger sequencing (data not shown). Neither AcLEF-2^{C84A} nor AcLEF-2^{C84A}/Hi5 p1 contained a mutation inside *fp25k* when compared to wt AcMNPV (NCBI, Accession no NC_001623). Both viruses encoded for a 214 amino acid FP25 protein.

8.4 BV production of AcLEF-2^{C84A}/Hi5 cells

A characteristic of a persistent virus infection is the continuous production of BVs. To determine BV production in AcLEF-2^{C84A}/Hi5 cells, cell culture media from confluent monolayer cells from different passages was harvested and the virus was titrated by plaque assays (Fig. 8.2). In all analysed passages of AcLEF-2^{C84A}/Hi5 cells BV was produced ($>10^3$ pfu/ml) although the virus titre varied.

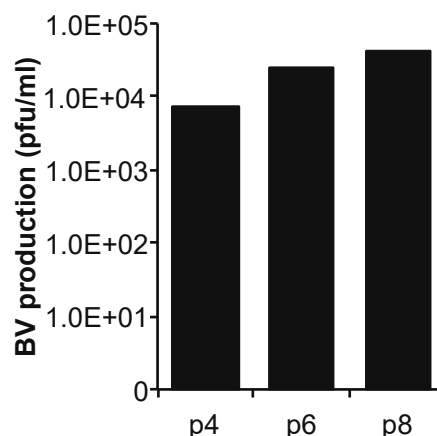


Figure 8.2 BV production of AcLEF-2^{C84A}/Hi5 cells

Cell culture medium from different passages (p) of confluent AcLEF-2^{C84A}/Hi5 cells was harvested and virus titre was determined by plaque assays (in pfu/ml).

8.5 AcLEF-2^{C84A}/Hi5 cell viability

To analyse the growth pattern and the viability of the newly established AcLEF-2^{C84A}/Hi5 cell line, 10⁶ cells/ml of passage p9, p11 and p12 with a viability of 90.9%, 89.3% and 98.3%, respectively were seeded into 25 ml shake culture and incubated at 28°C at 135 rpm. The amount of cells was counted daily by light microscopy using Trypan Blue staining (Invitrogen™) and a Neubauer counting chamber to determine the number of dead cells. Cell growth pattern and viability were compared to *T. ni* Hi5 cells (p69, 96.2% viability at day 0).

The parental *T. ni* Hi5 cells showed a typical growth curve containing a log phase, a diminishing growth phase and reaching a maximum of live cells 3 d p. seeding (>6x 10⁶ cells/ml; Fig. 8.3). Almost 100% of *T. ni* Hi5 cells were viable until the growth curve peaked but subsequently viability decreased dramatically. At 7 d p. seeding all cells were dead.

The AcLEF-2^{C84A}/Hi5 cells (as seen for p12; Fig. 8.3) initially resembled a similar growth curve as observed for its parental cell line undergoing a log phase, reaching a maximum of live cells (~2.5x 10⁶ cells/ml) at 3 d p. seeding until the number of live cells decreased until day 6. Even its viability initially resembled those of its parental cell line. After day 6 cells of p12 entered a stationary phase (15-20% viability) for about 6 days before their viability started to decrease. The number of live cells of p11 and 12 decreased after transferring cells from monolayer into suspension culture from ~90% (day 0) to ~65% (day 1; Fig. 8.3). Subsequently, the viability of AcLEF-2^{C84A}/Hi5 cells from p11 recovered slightly. Their number of live cells was highest at 3 d p. seeding (only ~10⁶ cells/ml) before the number and their viability slowly decreased over the observed period of time.

However, the number of live cells of p11 did not increase. On the contrary, their viability and the amount of live cells slowly declined over time. Unfortunately, determining the growth curve and viability of AcLEF-2^{C84A}/Hi5 cells had to be interrupted although not all of the cells were dead at the latest time point analysed (17 d p. seeding). Although the number of live cells was similar in all AcLEF-2^{C84A}/Hi5 passages analysed at 17 d p. seeding ($\sim 10^5$ cells/ml), their viability ranged from <5% (p12) to $\sim 25\%$ (p11; Fig. 8.3).

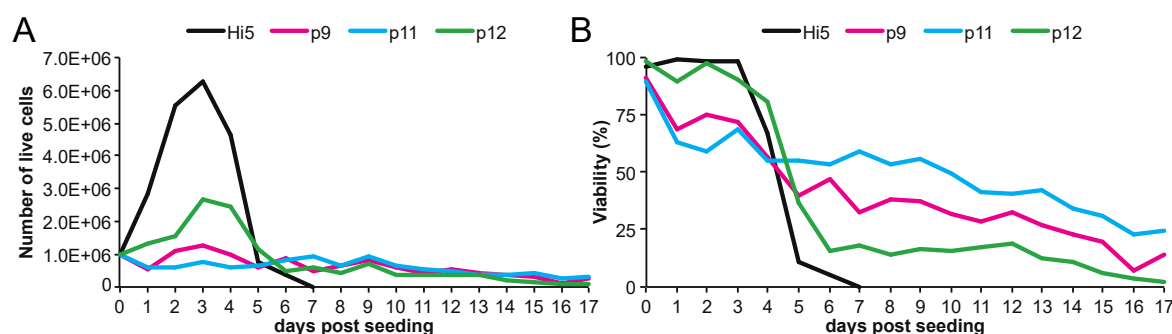


Figure 8.3 Growth curve and viability of AcLEF-2^{C84A}/Hi5 cells

10^6 AcLEF-2^{C84A}/Hi5 cells/ml passage (p) 9, p11, p12 and as a control its parental cell line *T. ni* Hi5 (Hi5; p69) were seeded in 25 ml suspension cultures and incubated at 28°C at 135 rpm. Cells were counted daily using Trypan Blue staining (Invitrogen™). A) The number of live cells and the B) viability of each passage and the control cell line was plotted against time.

At the time of writing the AcLEF-2^{C84A}/Hi5 cell line still survives and has been cultivated for over five months (since 11th April 2018) and was passaged until p17.

8.6 Discussion

The aim of this chapter was to establish a persistent baculovirus infection in *T. ni* Hi5 cells and to initially characterise the newly generated cell line. Previously, it was impossible to recreate an insect cell line harbouring a persistent baculovirus infection. Even the C20 cell line could not be regenerated (R. Arinto Garcia, R. D. Possee, personal communication). It was speculated that an enhanced BV production was the reason for this. Therefore, the virus AcLEF-2^{C84A} (Chapter 4) having an impaired BV production was utilised for the establishment of the persistent cell line AcLEF-2^{C84A}/Hi5 (Fig. 8.1C-F). It would be of interest to determine if other viruses generating a lower titre of BV would be able to establish a persistent virus infection in *T. ni* Hi5 cells. In addition, it may be hypothesised that the down regulation of BV production via the inhibition of virus structural protein synthesis (e.g. *vp39* or *gp64*) by RNAi might be applicable to permit a persistent baculovirus infection.

While the persistently infected cell line was established the cells underwent a crisis stage and some subcultures suddenly changed to a lytic infection (data not shown). This indicated a very fine balance between the host and the virus, which can be easily

disrupted although it is still unknown how this shift occurs. Also C20 cells went through a crisis stage especially during the early passages. However, the longer and the more often the C20 cells were subcultured the more robust they got suggesting an adaptation from the virus and the host (R. Arinto Garcia, R. D. Possee, personal communication).

During the subculturing of the AcLEF-2^{C84A}/Hi5 cell it was noted that cells produced a FP phenotype from a very early passage (Fig. 8.1E, F). It could be confirmed that AcLEF-2^{C84A}/Hi5 from at least passage 13 contained the most common *fp25k* mutation, which is an A insertion within seven existing As resulting in a truncated FP25K (data not shown; Kelly *et al.*, 2006). Interestingly, neither the virus stock utilised for the generation of AcLEF-2^{C84A}/Hi5 nor passage 1 of the persistently infected cell line contained a *fp25k* mutation indicating that the virus genome underwent changes and evolves. However, Sanger sequencing, the method utilised, is not sensitive enough to detect if a minor subpopulation of virus stock already contained the analysed mutation. Deep sequencing (e.g. Illumina® NGS platforms) could provide an answer to this question. The same *fp25k* mutation as in passage 13 was also observed for viruses generated by C20 cells (R. Arinto Garcia, R. D. Possee, personal communication). However, AcLEF-2^{C84A}/Hi5 p13 virus might contain additional mutations inside *fp25k*, which could not be confirmed as the persistently infected cell line contained a mixed population of virus. Subcloning of *fp25k* into a vector would be required to determine the different *fp25k* mutations inside the various virus populations by Sanger sequencing. A *fp25k* mutation can also arise from the insertion of host DNA (Beames and Summers, 1989, 1990). Serial passaging of baculoviruses *in vitro* usually results in a *fp25k* mutation potentially because the generation of OBs is nonessential in cell culture (Knudson and Tinsley, 1974; Hink and Strauss, 1976). The FP mutants are defective in polyhedra production and have an increased production of BVs (Fraser and Hink, 1982; Harrison *et al.*, 1996). It may seem contradictory at first that the persistently infected insect cell lines had a mutation inside a gene that enhances BV production. However, other factors may also regulate BV production and may help the persistent virus to maintain (see Chapter 6, 7).

Nevertheless, the newly generated AcLEF-2^{C84A}/Hi5 cell line continuously produced BV in the early cell culture passages (Fig. 8.2). This indicated that AcLEF-2^{C84A} was present as a persistent virus infection. Furthermore, as BV production varied between passages a dynamic interaction and a fine balance between host and virus gene expression was maintained. However, if all viral genes were expressed or how viral replication was affected in AcLEF-2^{C84A}/Hi5 cells was not investigated. Production of BVs was also observed in all analysed passages of C20 cells with a trend of less BVs production in later passage (>p100; R. Arinto Garcia, R. D. Possee, personal communication). It would be interesting to investigate if this might be also true for AcLEF-2^{C84A}/Hi5 cells. In C20 cells

every single cell produced BV as determined by single cell dilution to establish the cell line (R. D. Possee, personal communication). If every cell of the AcLEF-2^{C84A}/Hi5 cell line generates BVs still needs to be examined. On the contrary, it was also observed that only one per cent of cells were infected during any subculturing when Sf cells became persistently infected with SfMNPV (Crawford and Sheehan, 1983).

Nevertheless, there was a difference in BV production in the early passages of AcLEF-2^{C84A}/Hi5 and C20 cells. While C20 cells generated $>10^8$ pfu/ml, BV production of AcLEF-2^{C84A}/Hi5 was reduced compared to C20 cells ($<10^5$ cells/ml; Fig. 8.3). The reason for this is probably the impaired BV production caused by the LEF-2^{C84A} mutation inside the AcMNPV genome.

Moreover, AcLEF-2^{C84A}/Hi5 cells in suspension were able to survive longer than its uninfected parental cell line (Fig. 8.3). Therefore, they were more robust and more resilient than *T. ni* Hi5 cells. Furthermore, the growth pattern of different passages of AcLEF-2^{C84A}/Hi5 cells varied and can resemble those of *T. ni* Hi5 cells. The robustness and resilience was also observed for C20 cells, which could survive up to 40 days in suspension culture without any subculturing. Unfortunately, the maximum time that AcLEF-2^{C84A}/Hi5 might remain viable in culture could not be determined in this study. Although C20 were viable for longer than *T. ni* Hi5 cells, their longevity varied between passages further indicating a dynamic relation between the virus and the host (R. Arinto Garcia, R. D. Possee, personal communication). Interestingly, the viability of AcLEF-2^{C84A}/Hi5 cells can decrease after seeding cells from monolayer into suspension culture. A possible reason for this may be that cells were stressed and although cells recovered a subpopulation may have changed into an overt infection. Stress such as temperature changed or UV light was observed to be required for activation of covert into an overt baculovirus infection *in vivo* (reviewed by Williams *et al.*, 2017). Also, C20 cells were not introduced to suspension cultures until they had been kept as monolayers for over 100 sequential passages (R. D. Possee, personal communication). Hence, they had had much longer to adapt to the persistent infection than AcLEF-2^{C84A}/Hi5, which had been in culture for a much shorter period.

One aspect that has not been investigated in AcLEF-2^{C84A}/Hi5 cells is if these are susceptible for superinfection. Only a minor subpopulation of C20 cells was infected with a second AcMNPV (Chapter 6; R. Arinto Garcia, R. D. Possee, personal communication). If AcLEF-2^{C84A}/Hi5 cells were resistant to superinfection, it would be interesting to determine if the blockage for a second virus infection occurred as early as during virus binding as in C20 cells or at a different step during the viral replication cycle.

8.7 Conclusion

The aim of this chapter was to generate a *T. ni* Hi5 cell line persistently infected with a baculovirus. This could be achieved utilising the AcLEF-2^{C85A}, which contains an impaired BV production. The initial characterisation of the newly established AcLEF-2^{C84A}/Hi5 cell line determined similarities to the C20 cell line. The characteristic of a persistent virus infection was achieved as BVs were continuously produced. Moreover, AcLEF-2^{C84A}/Hi5 cells are very robust and resilient and live longer than their parental *T. ni* Hi5 cell line. Additionally, a FP phenotype was observed resulting from mutations inside the *fp25k* gene of the persistent baculovirus.

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Chapter Nine:

Final Discussion

Baculoviruses and their gene expression have been an increasing topic of research interest since they were first successfully applied as expression vectors for recombinant proteins (reviewed by van Oers, 2011; Rohrmann, 2013). In contrast to other protein expression systems (e.g. *E. coli*, adenoviruses, lentiviruses etc.), the insect-specific baculoviruses (mainly AcMNPV) offer several advantages, e.g. they are safe for humans, they can produce very high yields of recombinant proteins, recombinant proteins get posttranslational modified, the manufacturing process is simple and fast and the production of recombinant proteins can be scaled up easily (see Introduction).

Nowadays the BEVS has been commercialised for the production of recombinant vaccines. However, although improvements have been made since the first publication describing the production of human IFN- β under the *polh* promoter (see Introduction 1.7; Smith *et al.*, 1983b), the BEVS still has limitations: The main one being that the *in vitro* system results in the death and often lysis of the virus-infected cells. Therefore, recombinant protein expression by the BEVS requires the constant infection of fresh cell cultures in a batch process. Additionally, the expression levels between individual proteins can vary significantly. The reasons for variations are still not fully understood (see Introduction for more details). Therefore, the aim of this thesis was to determine how late and very late gene expression in baculovirus infections are regulated. As baculoviruses can either cause cell lysis at the end of their replication cycle or can establish a persistent infection both overt and covert viral gene expressions were analysed. Moreover, an *in vitro* system of persistently infected cells with a baculovirus was utilised to determine the factors required for the regulation of viral gene expression and how this is distinguished to a lytic virus infection. The results obtained will eventually further improve the BEVS.

Traditionally, recombinant proteins are expressed under the strong nonessential very late *p10* or *polh* promoters (Smith *et al.*, 1983b; Knebel *et al.*, 1985; Vlak *et al.*, 1990). Historically, before the invention of baculovirus knock-out systems (Luckow *et al.*, 1993), *p10* and *polh* expression studies were often performed using reporter genes, e.g. *lacZ* (Knebel *et al.*, 1985; Vlak *et al.*, 1988; Ooi *et al.*, 1989; Qin *et al.*, 1989; Min and Bishop, 1991; Roelvink *et al.*, 1992; van Oers *et al.*, 1992). Hence, initially, native P10 and polyhedrin accumulation of virus-infected cell lysates was studied on Coomassie-stained protein gels (Chapter 3). Both very late proteins accumulated in wt-infected *Sf9* and *T. ni* Hi5 lysates from 48 h p.i. although polyhedrin accumulated to higher levels than P10 in both insect cell lines analysed. Despite the fact that both promoters contain AT-rich burst and essential leader sequences (Matsuura *et al.*, 1987; Possee and Howard, 1987; Weyer and Possee, 1988, 1989), distinct temporal expression profiles for *p10* and *polh* were observed (Vlak *et al.*, 1988; Ooi *et al.*, 1989). Expression of the very late genes occur between 12-18 h p.i. in virus-infected cells (Chapter 6; Weyer *et al.*, 1990). However, *p10*

is expressed earlier than *polh* and reached lower maximal transcription levels (Vlak *et al.*, 1988). For example, at 18 h p.i. the *polh* abundance is 1.5-2-fold higher than *p10* (Min and Bishop, 1991). Hence, *p10* may be rather considered as a delayed late gene. This stressed out the importance to study both *polh* and *p10* expression in this thesis.

To examine the regulation of AcMNPV gene expression the effect of candidate genes (*cath*, *chitA*, *lef-2* and *vp39*) on very late gene expression and the global regulation of gene expression in both lytic and persistent baculovirus infections was investigated. The methods utilised in this study include recombinant viruses either generated by specific gene knock-outs, site-directed or random mutagenesis, RNAi and strand-specific (Illumina®) RNA-seq.

An interesting early observation in this study was that P10 appeared to be cleaved (Chapter 3; Weyer *et al.*, 1990). Processing of the perinuclear P10 by Cath was crucial to initialise nuclear lysis and polyhedra release although the physical process of how P10 is involved in the breakdown of the nuclear membrane remains elusive. As a consequence of cell lysis, the colloid osmotic pressure decreases forcing water into the nucleus. This induces swelling of the nucleus and nuclear membrane stretching (reviewed by Enyedi and Niethammer, 2017). The perinuclear P10 (“cage structure”) stabilises the nucleus of virus-infected cell, protects against early nuclear lysis and therefore against the release of premature polyhedra (Carpentier *et al.*, 2008).

In general, the nuclear envelope breakdown during mitosis requires the disassembly of nuclear lamin, which underlies the nuclear envelope. This is caused by phosphorylation by Cdc2. The nuclear pore complexes dissociate into subunits, which is also a result of phosphorylation. Moreover, dynein was implicated to trigger tearing of the nuclear envelope by mechanical forces (reviewed by Gönczy, 2002; Hetzer, 2010). It remains to be speculated that a structural change of P10 may facilitate nuclear breakdown by mechanical forces from the outside of the nucleus, which may be supported by the following observations: a) At 96 h p.i. the P10 “cage” structure changes into a thick polarised P10 mass; b) a minimal P10 concentration was required for nuclear lysis to occur (Graves, 2017) to potentially accumulate a minimal force required for nuclear lysis. This would further explain why *p10* needs to be hyperexpressed.

The main method to analyse the function of *cath*, *chitA* and *p10* in nuclear lysis involved the utilisation of knock-out mutants and their effects on protein accumulation, which was visualised by protein gels. Although it was determined that no functional proteins were made in the null mutant viruses, it was not confirmed that the effects observed were due to the gene of interest. No rescue viruses were generated to test for the reversal

phenotype. That controls are absolutely necessary for the proper interpretation of the gained experimental data was shown by the study of AcMNPV LEF-2 (Chapter 4).

It could be determined that different domains of AcMNPV LEF-2 were required for DNA replication, while its role in very late gene expression is still arguable. To examine the role of LEF-2 both random and side-directed mutagenesis studies were utilised. It was proposed that LEF-2 was required for very late gene expression by the generation of VLD1 using BrdU mutagenesis. Unfortunately, the mutation predicted to be required for *polh* and *p10* expression (LEF-2^{D178N}) was not confirmed in the original work, e.g. by running cell lysates of AcLEF-2^{D178N}-infected insect cell on a protein gel. It was assumed that the mutation inside LEF-2 was crucial for very late gene expression only because BV production and DNA replication of VLD1 and AcLEF-2^{D178N}-infected cells was similar (Merrington *et al.*, 1996). However, this was a fallacy as it could be demonstrated in this study that LEF-2^{D178} did not affect very late gene expression and has if anything a supportive function (Chapter 4). Although sequencing techniques at the time during the performance of the initial study were not as advanced as they are nowadays, it demonstrates how crucial the adequate control experiments are in life science and in research in general.

Furthermore, this raises the question if random mutagenesis studies are still a suitable tool or if side-directed mutagenesis studies as also performed on LEF-2 (Chapter 4) should be preferred. Random mutagenesis studies have been a powerful tool to search for phenotypes after which the genotype of an organism and the protein function are concluded (Shortle *et al.*, 1979; Rechinsky *et al.*, 1993; Katsuma and Kokusho, 2017). However, as the name already implies it has the big disadvantage that mutations in various (random) locations of the genome can be inserted, which may in addition affect the phenotype (Hopkins and Goodman, 1980). An additive phenotype of *lef-2* and *pk-1* was hypothesised from the studies on VLD1 although it has not been proved yet (Chapter 4). Additive functions of AcMNPV genes have almost not been investigated apart from *fp25k* and *p35* (see 4.7; Kelly *et al.*, 2006). Therefore, there may be other so far undetected interplays between various viral or even host genes that determine a phenotype as a consequence of virus infection.

On the other hand, site-directed mutagenesis studies are performed to analyse a predicted phenotype from a known genotype. Even with the increased available bioinformatics tools it should be still asked how accurate the predictions are especially in a very complex system. Therefore, the suitable technique for a specific scientific question should be considered carefully beforehand. Additionally, the interpretation of data obtained from random mutagenesis studies should be taken with caution.

To also gain a more global picture on the regulation of gene expression *in vitro* strand-specific RNA-seq was performed either to analyse the effects of a *vp39* knock-down on virus gene expression (Chapter 5) or to compare the regulation of gene expression of a covert with a lytic infection (Chapter 6, 7).

As analysed, *vp39* RNAi did not only down regulate very late gene expression but furthermore differentially regulated 29 viral genes (Chapter 5). Even other baculovirus structural proteins such as VLF-1 or P6.9 seem to be required for the hyperexpression of very late genes (McLachlin and Miller, 1994; Yang and Miller, 1999; Li *et al.*, 2015). A strict categorisation of viral genes into structural, regulator and accessory groups (Chapter 6) may therefore understate their function. Moreover, a global effect on viral gene expression was also observed after insect cells were infected with a *pe38* or *pp31* knock-out virus (Jiang *et al.*, 2006; Yamagishi *et al.*, 2007). Hence, viral gene expression is probably regulated in a transcriptional network resulting in the characteristic temporal phases of gene expression (Chapter 6), which additionally regulate and is regulated by host gene expression (Chapter 7). Silencing of a single gene may therefore have various unpredicted effects on the entire transcriptional network and may change several feedback mechanisms. Our view of gene function still tends to follow the idea that one gene has one purpose. This is probably completely erroneous and ignores the fact that organisms are extremely resilient and probably have multiple redundancies built in. Even the deletion of *lef-2* was unable to prevent all virus replication (Chapter 4), suggesting that viruses can survive even quite catastrophic modifications to their genomes.

As a method for the down-regulation of gene expression the RNAi system was chosen, which is commonly accepted since it was first discovered in the model organism *Caenorhabditis elegans* 20 years ago (Fire *et al.*, 1998). Nevertheless, this technique contains some hurdles, which includes the lack of specificity and off target activity of the dsRNA. Short dsRNAs may cause direct silencing of nontargeted genes containing as few as eleven contiguous nucleotides identity to the siRNA. Moreover, genes involved in various cellular pathways can be nonspecifically repressed or even stimulated (Jackson *et al.*, 2003; Persengiev *et al.*, 2004; Scacheri *et al.*, 2004). To manage off target activity the effect of a second or better third non-overlapping siRNA targeting the same gene of interest should have been analysed to verify the observed phenotype. Additionally, the lowest effective concentration of siRNAs should be used to minimise any nonspecific effects (Persengiev *et al.*, 2004). For the *vp39* knock-down 5 µg of dsRNA for a 24 well format was utilised (Y. Guo; Chapter 5) although as little as 500 ng for a 6 well plate is sufficient for the successful knock-down of other viral structural genes (data not shown; R. D. Possee, personal communication). Also, the expression levels of other unrelated

(“housekeeping”) genes such as actin or GAPDH should have been determined to analyse for any unspecific effects of the dsRNA (e.g. by immunoblotting).

A more recent but more importantly a more specific and precise technology to edit DNA is the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) system, which is a RNA-guided endonuclease based genome editing technology (Jinek *et al.*, 2012). Although it was shown to be highly robust and effective it can also induce off target mutations (reviewed by Peng *et al.*, 2016). However, they can be minimised by the utilisation of a high fidelity Cas9 (Kleinstiver *et al.*, 2016). The CRISPR/Cas technology was successfully applied in *Sf9* and *T. ni* Hi5 cells to modify the glycosylation pathway (Mabashi-Asazuma and Jarvis, 2017). It may be a valuable tool in the future to optimise host gene expression pathways in the BEV system and thereby improving the quality of recombinant proteins, e.g. to prevent their degradation or to optimise their folding.

The analysis of the RNA-seq data furthermore revealed that the persistent baculovirus lacked the typical temporal regulation of virus gene expression and did not enter the very late phase of infection (Chapter 6). Therefore, the cells may not die as a reason of virus infection. Moreover, the host transcriptome response altered from a lytic virus infection potentially caused by the evolution of the host together with the virus during the maintenance of the cell line (Chapter 7). However, to find differences and similarities in gene transcription in covert and overt infections *in vitro* it was decided to compare expression profiles as it is difficult to directly compare RPKM values of different conditions (see Chapter 6 for details). An alternative approach would have been to normalise gene expression values with a housekeeping gene or even with the geometric mean of several selective housekeeping genes to maximise the robustness of the test as already performed for RT-PCRs (Vandesompele *et al.*, 2002). However, transcription of various insect cell housekeeping genes (e.g. actin, GAPDH, tubulin or myosin) only stayed constant during the early virus infection (up to 24 h p.i.), after which expression decreases (Salem *et al.*, 2014). Hence, the reliability to use housekeeping genes as a reference can be doubted.

In general, covert baculovirus infections are more relevant *in vivo* and are rather artificial in cell culture. Persistent baculoviruses are present in most field collected *M. brassicae* and were also reported to be present in other insects, e.g. *S. exigua*, *S. exempta*, *C. fumiferana* (Burden *et al.*, 2006; Vilaplana *et al.*, 2010; Kemp *et al.*, 2011; Murillo *et al.*, 2011). A persistent baculovirus infection in natural populations allows a fitness advantage for the virus to ensure survival when host populations are low or when horizontal transmissions are restricted (reviewed by Williams *et al.*, 2017). Although C20

cells represent an artificial system they are the best possible system to study covert infections in more detail so far. *In vivo*, stress factors such as overpopulation, physiological stress conditions or temperature change may induce an overt infection (Fuxa *et al.*, 1999; Virto *et al.*, 2017; reviewed by Williams *et al.*, 2017). Hence, it may be challenging to analyse virus persistency under laboratory conditions *in vivo*. Although it would be interesting to determine how gene expression of a covert baculovirus infection *in vitro* shows similarities or differences from an *in vivo* situation. However, it may be difficult to analyse covert infections from field-collected insects by RNA-seq as gene expression may be under the detection limit. Even in a lytic baculovirus infection *in vivo* not every columnar and regenerative midgut cells gets infected (Keddie *et al.*, 1989). Moreover, viral genes of a lytic baculovirus infection *in vivo* only received a very low number of mapped reads as determined by Illumina® RNA-seq analysis (A. Shrestha and G. W. Blissard, personal communication).

Nevertheless, the predefined *in vitro* system may be utilised to determine how the transition between covert and overt baculovirus infections occur. If it was possible to determine the molecular switch between persistent and lytic virus infections this may be applied to improve pest control by baculoviruses.

Moreover, the possibility of reestablishments of new insect cell lines harbouring a persistent baculovirus (Chapter 8) will hopefully have beneficial effects on the BEVS. New cell lines could be generated, in which the recombinant protein of interest will be encoded under a strong baculovirus promoter. This may allow the expression of recombinant proteins for long-term without the need of reinfection of fresh cell cultures. A suitable promoter may be *p6.9* as in C20 cells the late *p6.9* transcripts were the most abundantly mRNAs present at all analysed time points (Chapter 6).

Furthermore, the establishment of persistent virus infections in a predefined system offer the chance to analyse virus-host co-evolution in more detail. An interesting question to answer in this aspect would be how virus and host adaption occurs inside a single population. There is evidence that baculovirus replication- and transcription associated genes and structural genes undergo positive selection (Hill and Unckless, 2017). Understanding virus-host evolution may also be of benefit to predict the virulence of emerging viruses (e.g. seasonal flu).

Historically gene expression analyses were performed by Northern blotting. With the improvements of bioinformatics tools the analyses changed from a single gene analysis to a more global transcriptome analysis using DNA-microarray techniques or more recently RNA-seq techniques (Chapter 5-7). In contrast to DNA-microarrays, RNA-seq provides several advantages such as higher specificity and sensitivity and the ability to detect new

transcripts. Especially when strand information is preserved, RNA-seq is a very powerful tool providing precise measurements about the levels of transcripts and the complexity of the transcriptome by parallel deep sequencing. However, the technique used in this study only provided a snapshot of the virus and host transcriptome. It does not take translational regulation (e.g. by mRNA structures), posttranslational modifications or protein turnover into account. Even small non-coding RNAs, such as host miRNAs may contribute to the posttranscriptional regulation of viral genes (Kang *et al.*, 2018).

An interesting example, which illustrates how quickly bioinformatics approaches have developed over time is the human genome project. The human genome project was proposed in the 1980s. This was just a few years after the first genome was sequenced completely, which belonged to the bacteriophage ϕ X174 (Sanger *et al.*, 1977). The sequencing project started in 1990 by an immense collaboration between international scientists and this extraordinary project took 15 years to accomplish with expenses of \$3 billion (Lander *et al.*, 2001). During the time and after finishing this giant project, the price for genomic sequencing dropped dramatically and in 2015 sequencing costs for a whole genome was below to \$1000 (Service, 2006; Phillips *et al.*, 2015). If the technology will improve as fast as during the last couple of years, it may be speculated that costs may decrease even down to \$100.

However, not only the price for NGS techniques decreased massively but also the sequencing speed and at the same time the genome coverage increased. While the initial sequencing of the human genome took years to be completed, nowadays it can be accomplished in hours (Illumina Sequencing Manual). In addition, while deep sequencing technology generating short reads by massively parallel sequencing was considered as revolutionised tool just a few years ago (reviewed by Wang *et al.*, 2009), single-molecule (nanopore) sequencing generating ultra-long reads have been developed more recently. This protocol generates read length up to 822 kbp ($N_{50}>100$ kbp) with a theoretical coverage of $\sim 30\times$ for the human genome (Jain *et al.*, 2018). Although single-molecule sequencing has significant higher error rates compared to Illumina® sequencing, the combination of nanopore sequencing followed by short-read corrections gives an assembly accuracy similar to Illumina® sequencing with the advantages of covering previous sequencing gaps and making *de novo* assemblies more tractable (reviewed by Chaisson *et al.*, 2015; Jain *et al.*, 2018).

Taking the massive and rapid improvements in combination with the decreasing costs of the NGS technologies into account it may be speculated that they will become a routine tool in every research and diagnostic laboratory, e.g. such as PCR techniques. In clinical diagnostic they may be routinely used for calling genetic variants, e.g. in oncology or for

germline variants in inherited diseases. However, the ethic aspects of their application in clinical (especially prenatal) diagnostics will not be discussed here.

Nevertheless, the improvements in NGS technology and bioinformatics required highly qualified staff for the processing and the interpretation of the massive amount of data. Hence, computer and natural scientist will need to collaborate even closer in the future to exploit all possibilities.

9.1 Conclusion

The complexity of gene expression networks and how they are regulated is still not fully understood although this study contributed to the knowledge of baculovirus gene expression regulation (e.g. verification of temporal phases of gene expression). However, recent advantages especially in the field of bioinformatics (NGS) but also in molecular biology (e.g. CRISPR/Cas) will further improve our knowledge. It will not only help to understand baculovirus gene expression even in more details for the improvements of recombinant protein expression but will also support our understanding of other infectious diseases. This will hopefully prevent epidemic and pandemic disease outbreaks in the future and may help to eradicate particular infectious diseases globally such as already seen for smallpox.

This thesis has contributed to several aspects of baculovirus replication and molecular biology. Although wide ranging in scope, their major impact may be in the routine development of cell lines that can continuously produce recombinant proteins owing to the use of vectors with curtailed infectious BV synthesis. Aspects of the work may also contribute to a final understanding of the role of the very late protein P10 in virus-induced cell lysis.

9.2 Future work

Although this thesis contributed to our knowledge about different aspects of baculovirus gene expression, we are still far away from completely understanding the whole complexity of baculovirus gene regulations.

Of particular interest might be to finally understand the role of P10 and P10 cleavage in virus-induced cell lysis. Although it could be determined that P10 could get specifically cleaved by Cath in the cell culture medium of virus-infected cells and that this processing is required for polyhedra release, it is still unclear where cleavage occurs. It would be interesting to examine the effect of *p10* recombinant viruses containing a point mutation inside the potential cleavage sites on nuclear lysis and polyhedra release.

Additionally, it was hypothesised earlier that Ac Δ chitA may delay nuclear lysis. Therefore, it might be worth quantifying nuclear disintegration to observe any differences between the recombinant and the wt viruses. One option how this was done before was by a quantitative LDH release assay (van Oers *et al.*, 1993; see discussion Chapter 3 for details on assay). Alternatively, released polyhedra could be quantified by flow cytometry. However, as different mutations inside P10 may influence its structure (van Oers *et al.*, 1993; Dong *et al.*, 2007; Raza *et al.*, 2017) confocal analysis should be undertaken to determine the structural integrity of the perinuclear P10.

Moreover, as P10 is highly structurally conserved it would be interesting to determine if cleavage of P10 is also conserved in closely related baculoviruses such as BmNPV or even in more distantly related ones such as SfNPV.

The role of the candidate gene *lef-2* in viral replication and very late gene expression was analysed in more detail. It could be determined that different domains of LEF-2 were essential for viral DNA replication and BV production. However, its specific role in very late gene expression is still unclear. We hypothesised that a combination of *lef-2* and *pk-1*, as found in VLD1, may be required for *p10* and *polh* expression. To confirm this hypothesis the very same mutations could be inserted into the AcMNPV genome to examine the effect on very late gene expression. It may be of interest to analyse the effects of the single point mutations, as found in VLD1 *pk-1*, on very late gene expression to determine the additive effects of *lef-2*.

Furthermore, the effect of the various recombinant *lef-2* mutants on very late gene expression has not been investigated at all. For example, it appears that VLD1-infected cells expressed the late protein P6.9 at higher levels than wt-infected cells (Fig. 4.3B). The basic protein P6.9, which runs on a protein gel at approximately 12.5 kDa is associated with the virus DNA in the virion nucleocapsid (Tweeten *et al.*, 1980).

In general, as LEFs play important roles during viral replication and are essential for late gene expression, all of them may be for candidate genes for the future analysis of very late gene expression.

VP39 was the last candidate gene analysed. The results obtained from this RNA-seq study are very preliminary as of massively overlapping transcripts and a more global effect on viral transcription after *vp39* RNAi. To investigate the involvement of *vp39* on very late gene expression the effect of both *vp39* and its upstream gene *cg30* have to be examined more closely although the generation of knock-out mutants may be difficult.

Additionally, it has not been analysed how a *vp39* knock-down or even more, how the transfection of a non-specific dsRNA may influence host gene transcription.

An interesting question in the context of the regulation of viral late and very late gene expression is, if host genes are involved. An analysis of the transcriptome response of AcMNPV-infected *T. ni* cells revealed a small subset (n=71) of host genes, which transcriptional level is low early during viral infection but become up regulated between 12-18 h p.i. (Chen *et al.*, 2014). This indicates they might play a role during the viral replication cycle. Hence, these host genes provide excellent targets, e.g. for a siRNA screen.

The second aspect of this thesis was to compare virus and host gene expression of a persistently infected cell line with a lytic baculovirus infection. Although mainly performed using bioinformatics approaches, it provides directions for further analyses. Of particular interest are the host gene pathways that altered in the C20 cell line compared to its parental cell line. For example the association of host cell chromatin with the persistently infected baculovirus could be further analysed by ChIP-sequencing. Alternatively, the involvement of the endocytic pathway might be examined by following virus entry cells and by co-staining with specific markers for the endosomal system, e.g. Rab5 for early endosomes or LAMP1 for lysosomes. Although it should be tested beforehand if commercially available markers for mammalian cells are also specific for insect cells.

Last but not least, this thesis may have an impact on the routinely generation of persistently-infected insect cell lines for recombinant protein expression. To follow up on this, the expression of various recombinant proteins under different promoters could be compared after the generation of newly covert *in vitro* systems. However, for the commercialisation of this system especially in vaccine production it might be of interest to cure insect cells from its persistent alphanodavirus TNCL.

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Appendix

Figure S 6.1-6.15 Virus gene transcription profiles in AcMNPV infected C20 and *T. ni* Hi5 cells

Normalised gene expression (RPKM) in AcMNPV-infected *T. ni* Hi5 (Hi5) and C20 cells were plotted over the time of infection. As mock-infected *T. ni* Hi5 cells were harvested 6 h p.s., unchallenged C20 cells from 6 h p.s. were considered as 0 h p.i.

Figure S 6.1 Most highly expressed AcMNPV genes in C20 mock 6 h

Figure S 6.2 AcMNPV genes encoding for structural BV proteins

Figure S 6.3 AcMNPV genes encoding for structural BV/ODV proteins

Figure S 6.4 AcMNPV genes encoding for structural ODV proteins

Figure S 6.5 AcMNPV genes encoding for structural OB proteins

Figure S 6.6 AcMNPV genes encoding for nucleocapsid proteins

Figure S 6.7 AcMNPV genes encoding for other structural proteins

Figure S 6.8 AcMNPV immediate early genes

Figure S 6.9 AcMNPV genes encoding for replicative proteins

Figure S 6.10 AcMNPV genes encoding for RNA polymerase and LEFs

Figure S 6.11 AcMNPV genes encoding for antiapoptotic proteins

Figure S 6.12 AcMNPV genes encoding for accessory proteins

Figure S 6.13 AcMNPV other genes-I

Figure S 6.14 AcMNPV other genes-II

Figure S 6.15 AcMNPV other genes-III

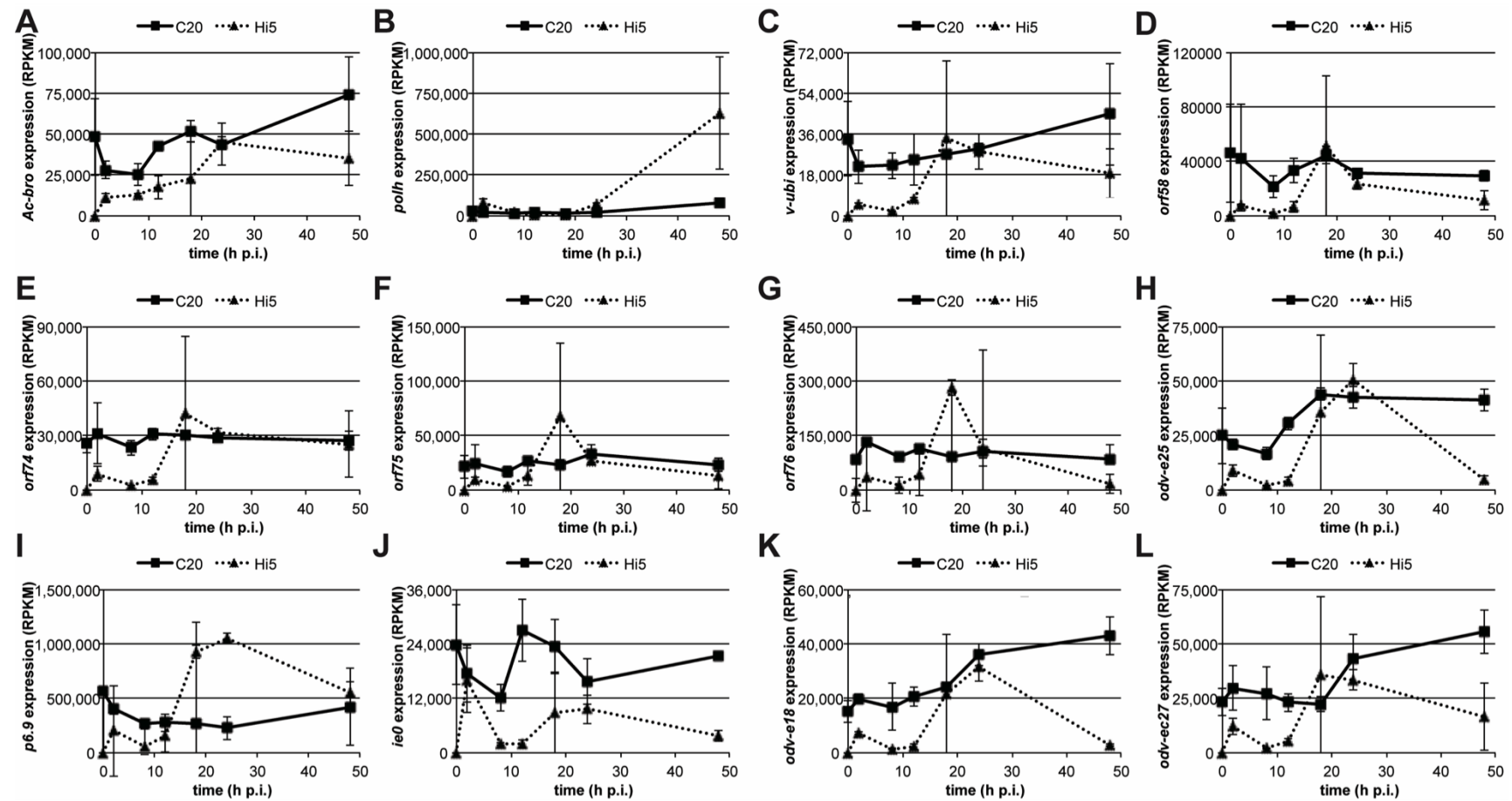


Figure S 6.1 Most highly expressed AcMNPV genes in C20 mock 6 h

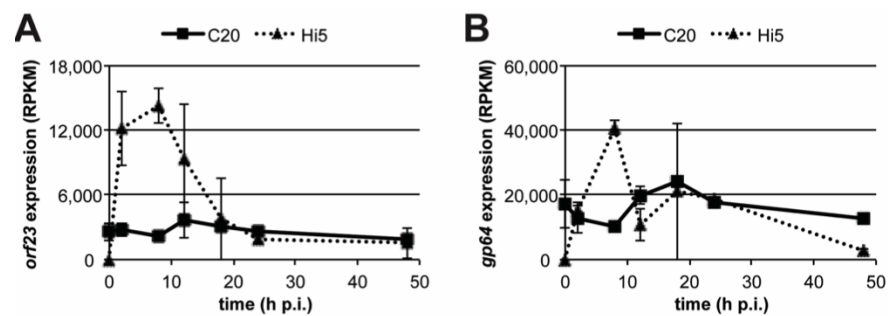


Figure S 6.2 AcMNPV genes encoding for structural BV proteins

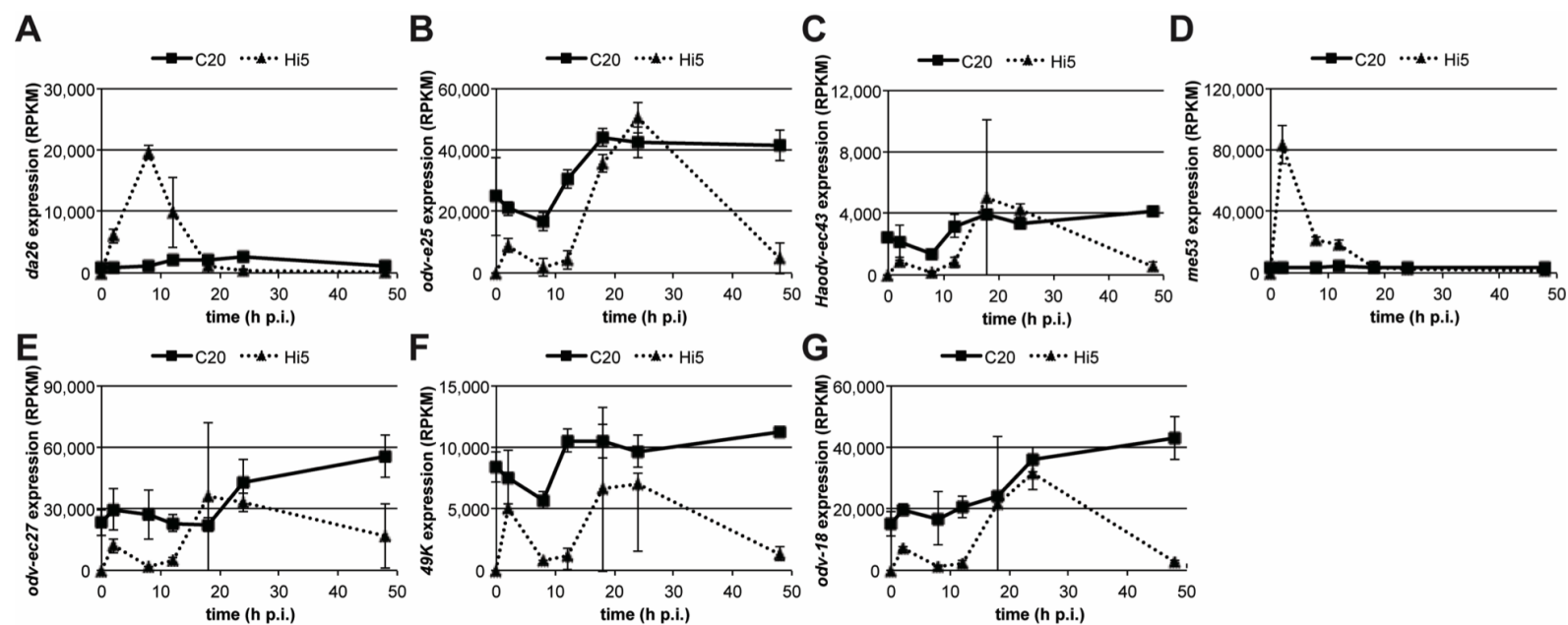


Figure S 6.3 AcMNPV genes encoding for structural BV/ODV proteins

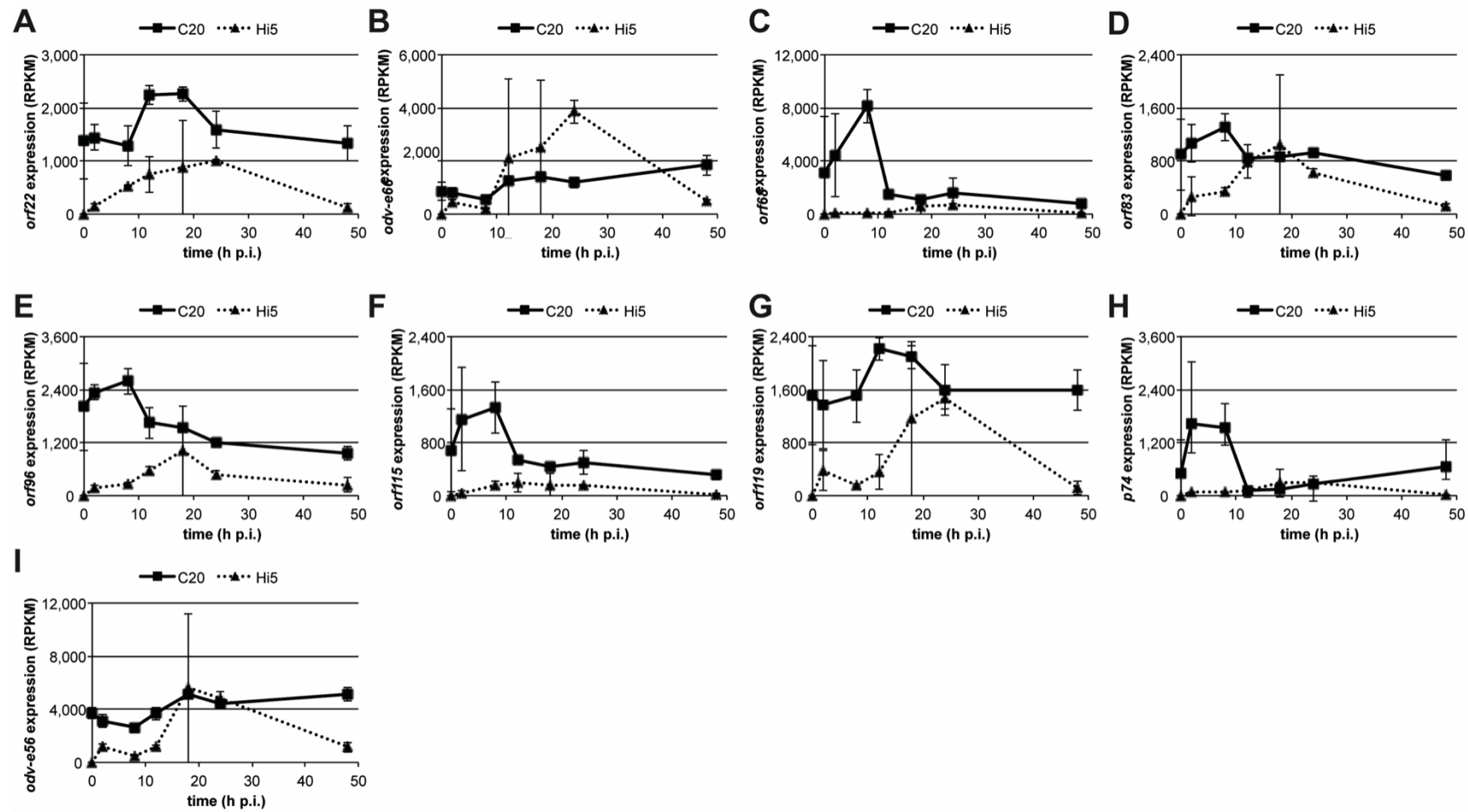


Figure S 6.4 AcMNPV genes encoding for structural ODV proteins

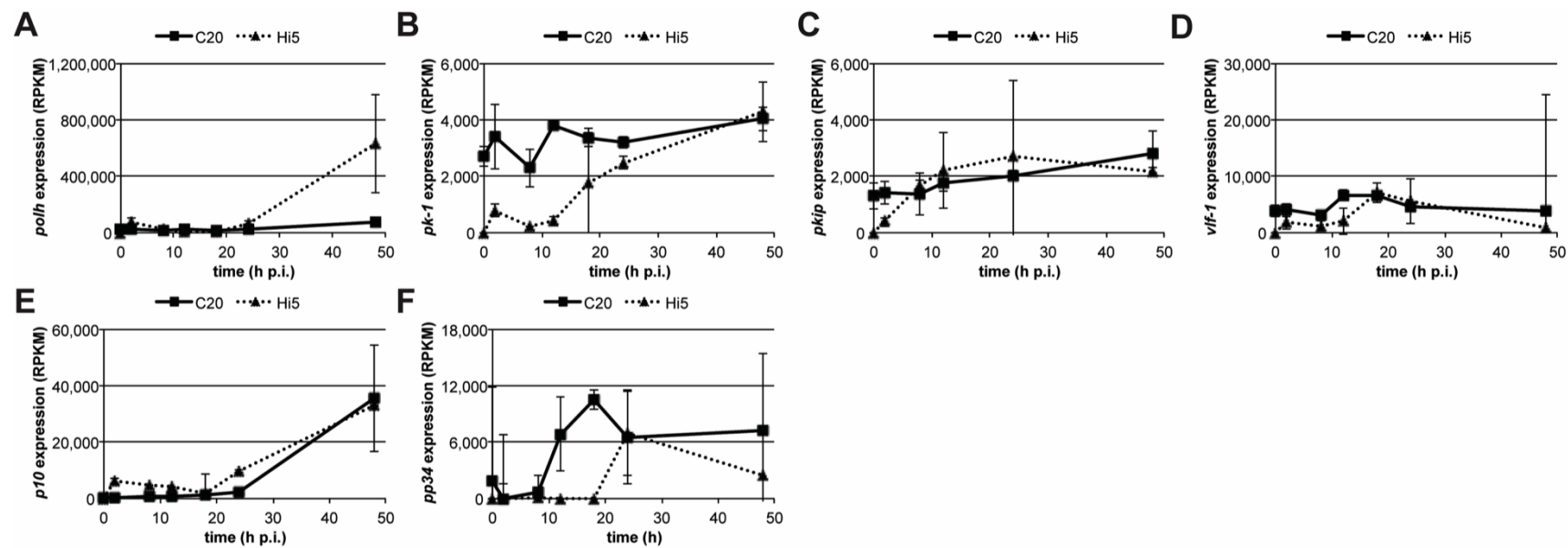


Figure S 6.5 AcMNPV genes encoding for structural OB proteins

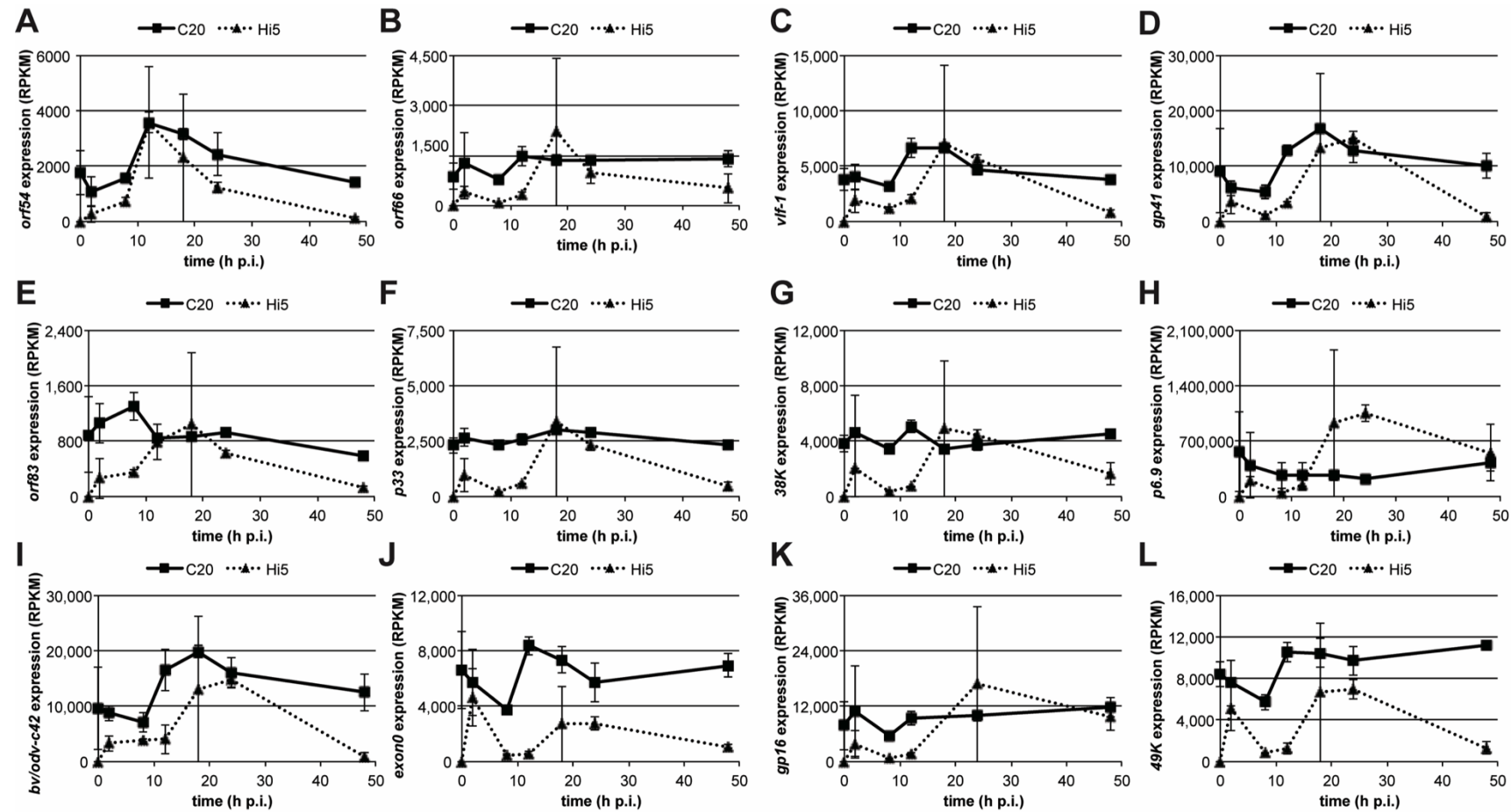


Figure S 6.6 AcMNPV genes encoding for nucleocapsid proteins

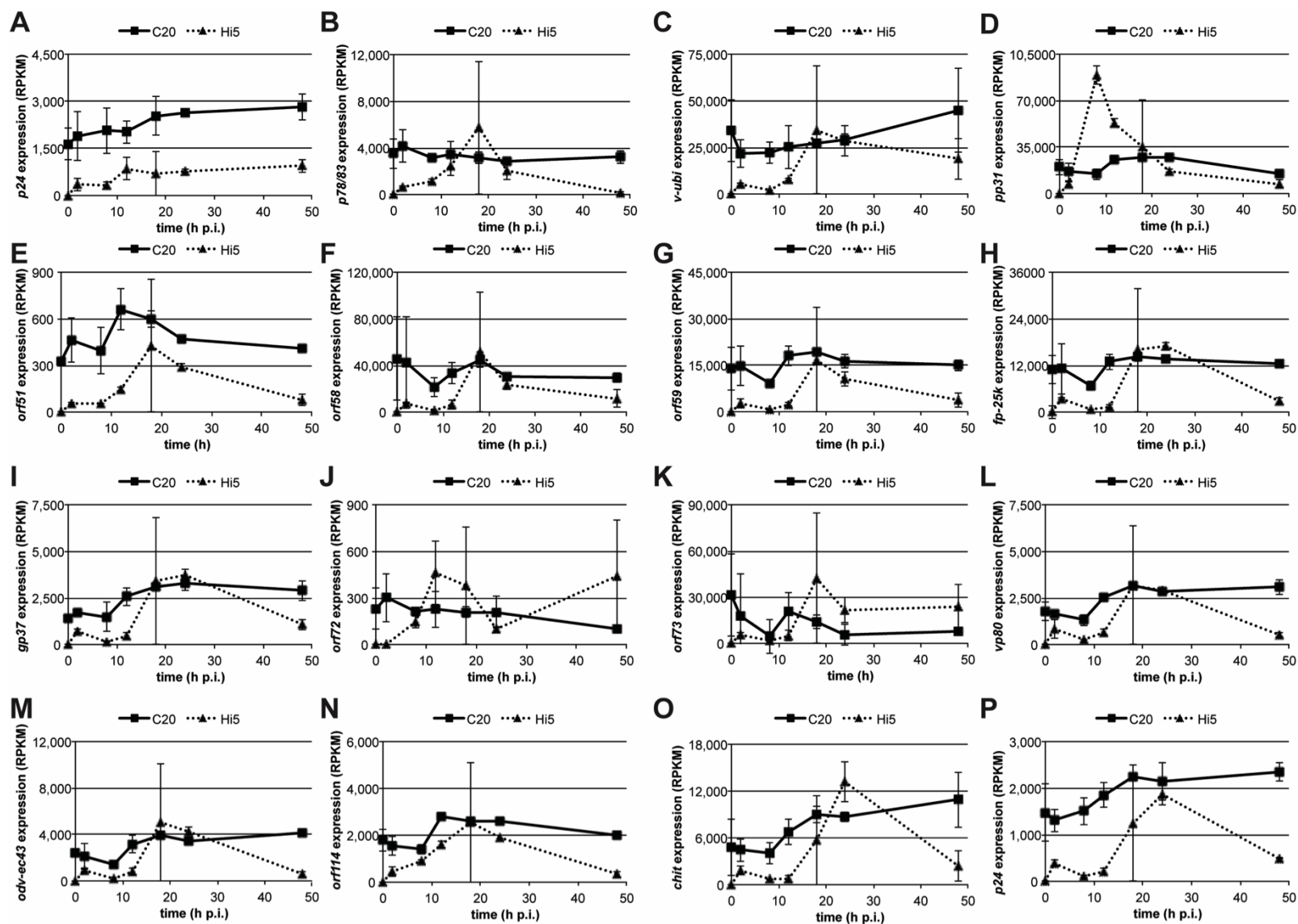


Figure S 6.7 AcMNPV genes encoding for other structural proteins

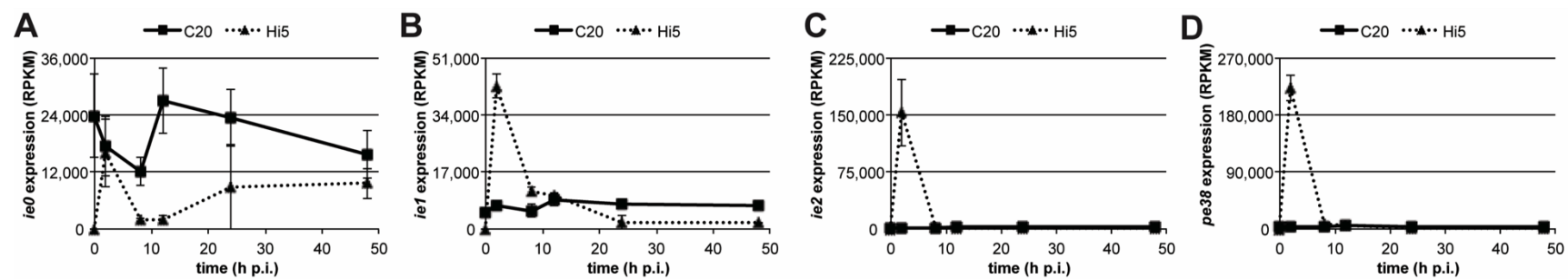


Figure S 6.8 AcMNPV immediate early genes

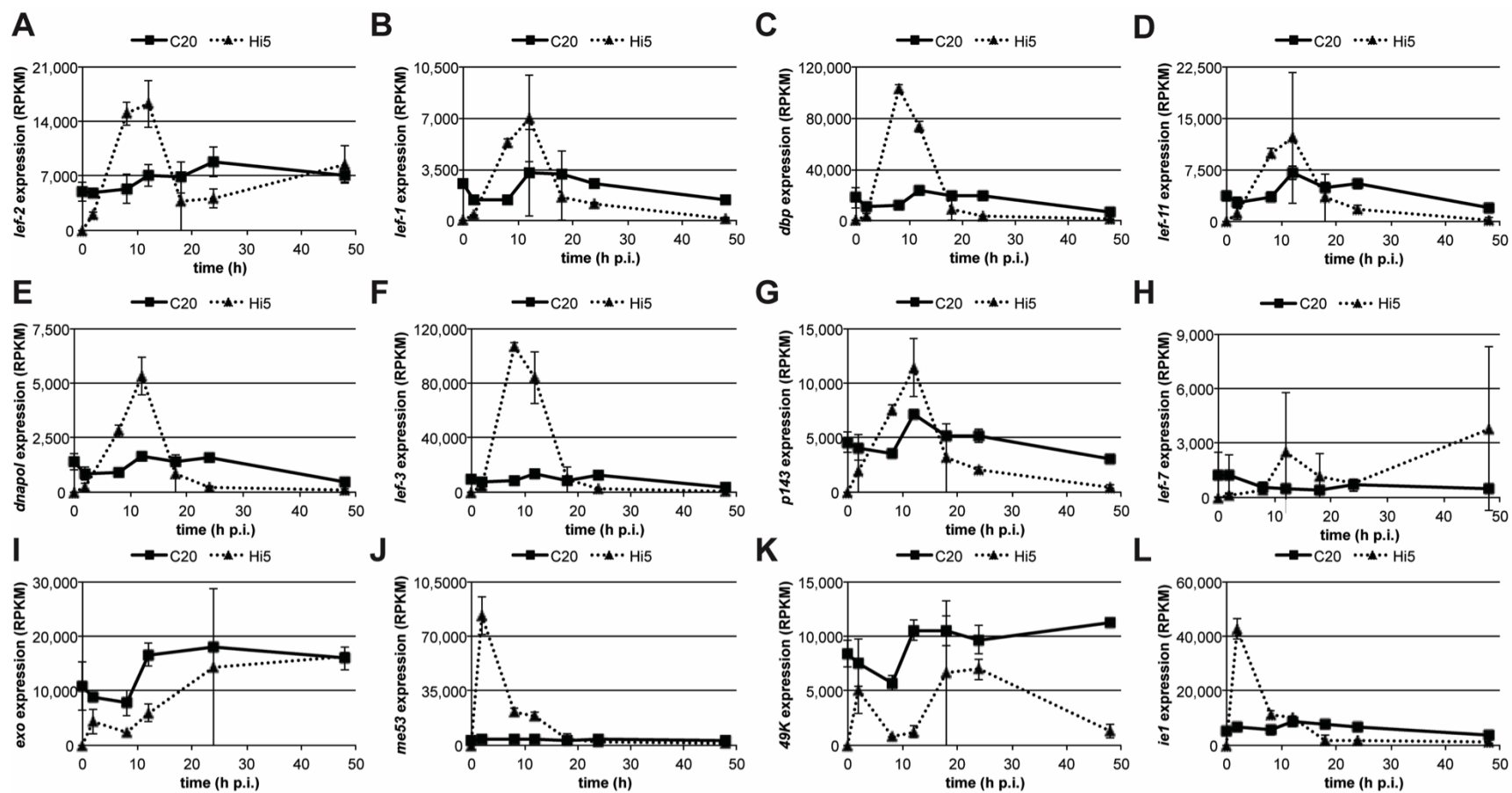


Figure S 6.9 AcMNPV genes encoding for replicative proteins

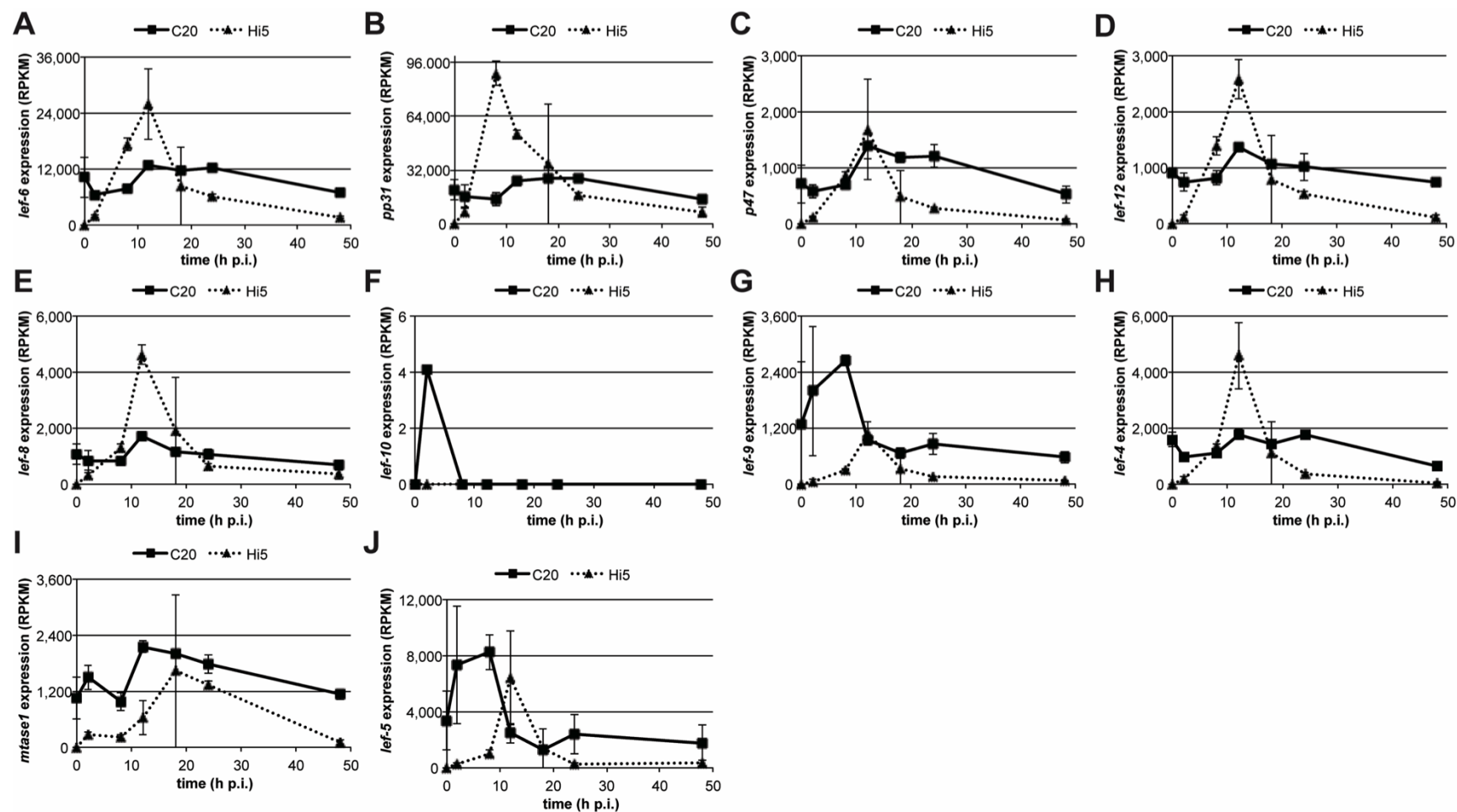


Figure S 6.10 AcMNPV genes encoding for RNA polymerase and LEFs

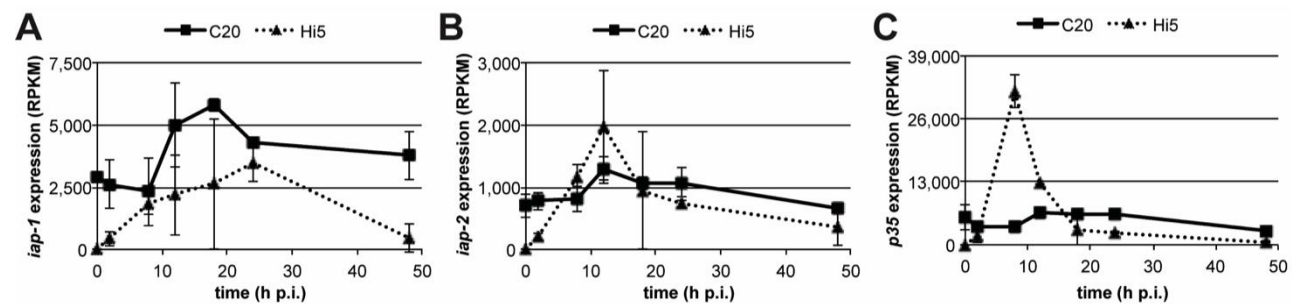


Figure S 6.11 AcMNPV antiapoptotic genes

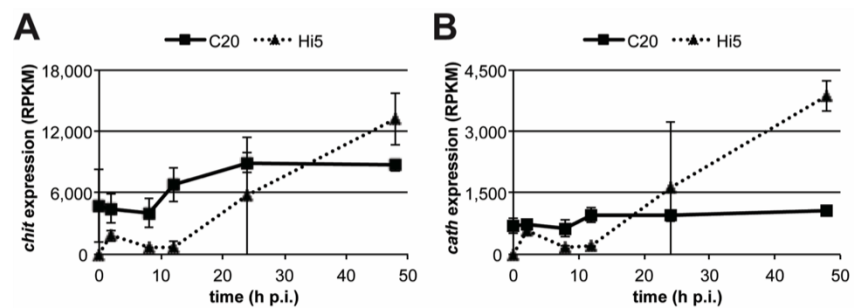


Figure S 6.12 AcMNPV genes encoding for accessory proteins

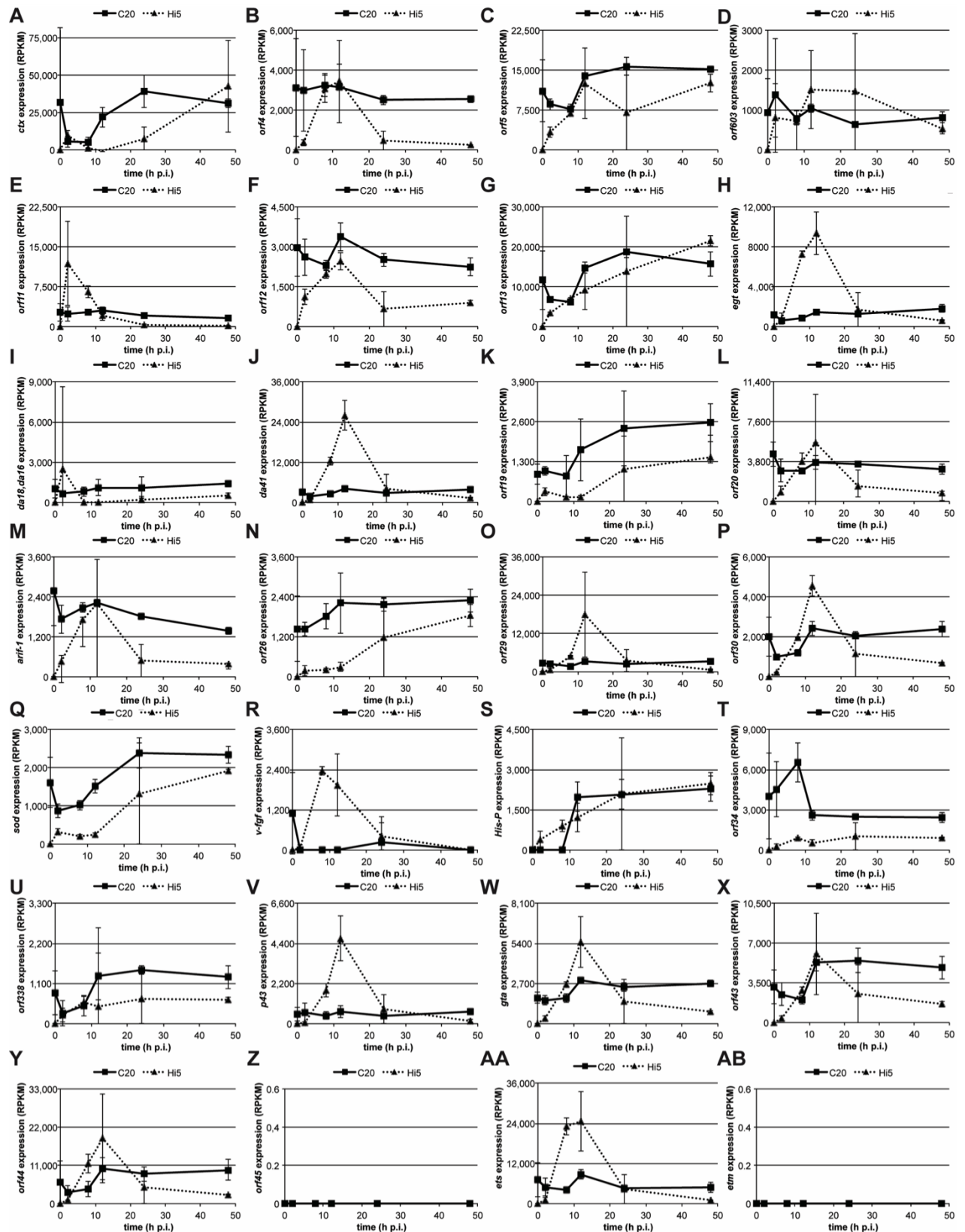


Figure S 6.13 AcMNPV other genes-I

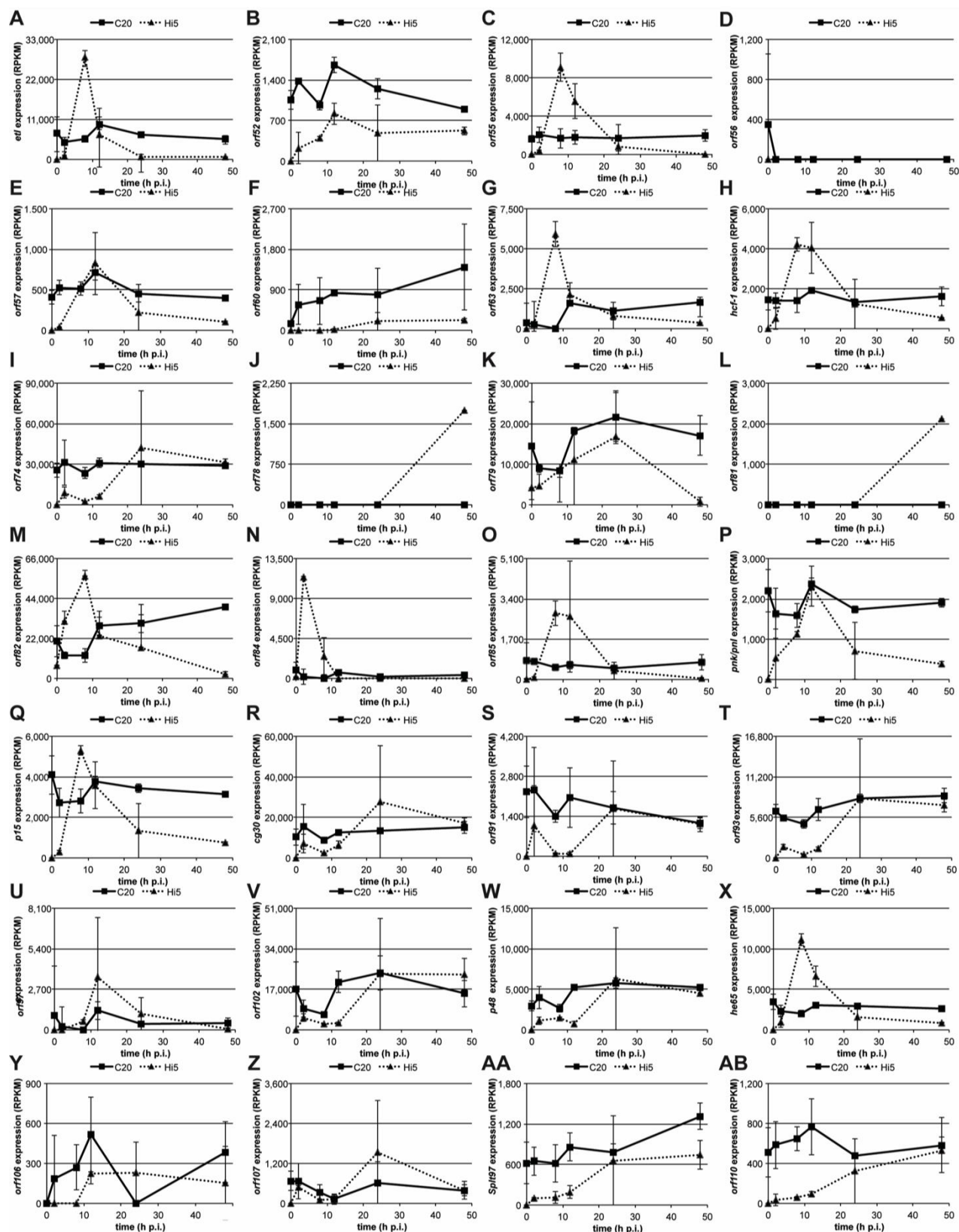


Figure S 6.14 AcMNPV other genes-II

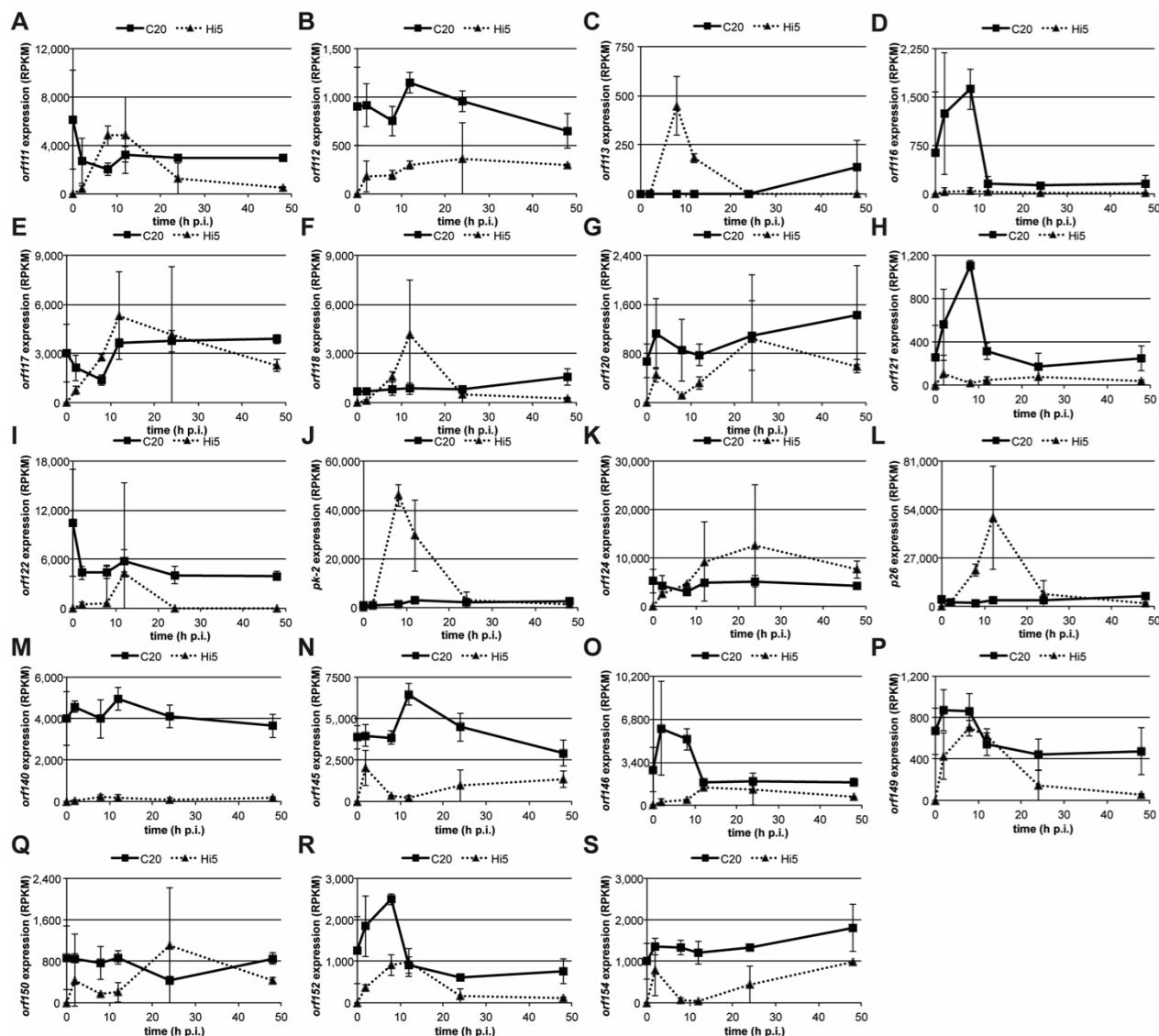


Figure S 6.15 AcMNPV other genes-III